HIERDIE EKSEMPLAAR MAG ONDER GEEN OMSTANDIGHEDE UIT DIE BIBLIOTEEK VERWYDER WORD NIE

University Free State

34300000100077
Universiteit Vrystaat

# *EXOPHIALA DERMATIDITIS* LIPASE: ISOLATION AND CHARACTERISATION

BY

**JOLEEN HAMILTON**

Submitted in the fulfillment of the requirements for the degree

**MAGISTER SCIENTIAE**

In the Faculty of Natural Sciences,
Department of Microbiology and Biochemistry, at the
University of the Orange Free State,
Bloemfontein

December 1998

Supervisor: Dr. A. van Tonder
Co-supervisor: Prof. D. Litthauer

Universiteit van die
Oranje-Vrystaat
BLOEMFONTEIN
- 9 MAY 2000
UOVS SASOL BIBLIOTEEK

# ACKNOWLEDGEMENTS

I sincerely wish to express my gratitude to the following persons and institutions that helped make this presentation possible:

Dr. A. van Tonder for his invaluable guidance, endless patience, encouragement and willingness to help. Without your inspiration this manuscript would not have been possible.

Prof. D. Litthauer for his guidance and willingness to help.

Prof. M.S. Smit, Andri and Elma Pretorius for the provision of the organisms which led to this research.

The Foundation for Research Development, my husband and parents for their financial support.

Monique Immelman for her help with the basic Microbiology skills.

Esta van Heerden and Bethuel Nthangeni for their help with the characterisation of the lipases.

Mr. P. Botes for his help with the preparation of this manuscript.

Fellow students from Biochemistry for being wonderful and helpful colleagues as well as friends.

My friends, especially Mariska du Plessis for their emotional support, encouragement and help.

My husband and parents for their unfailing support and love during all my years of study.

All the members of the Department of Microbiology and Biochemistry for interest shown and support.

Above all, God, my Creator, Father and Friend.

# CONTENTS

ACKNOWLEDGEMENTS i

LIST OF ABBREVIATIONS vii

LIST OF FIGURES viii

LIST OF TABLES xiii

CHAPTER 1 LITERATURE REVIEW 1

1.1 Introduction 1
1.2 Substrate specificity 2
1.2.1 Lipid class specificity 2
1.2.2 Positional specificity 2
1.2.3 Fatty acid specificity 3
1.2.4 Stereospecificity 3
1.3 Interfacial activation 3
1.4 The catalytic site 8
1.5 Kinetics of lipase 10
1.6 Lipase activity determination 11
1.6.1 Spectrophotometry 13
1.6.2 Fluorescence 14
1.6.3 Plate assays 14
1.6.4 Titrimetry 15
1.6.5 Controlled surface pressure 15
1.6.5.1 The monolayer technique 15
1.6.5.2 The oil drop method 16
1.6.6 Other assays 18
1.7 Purification procedures 18

1.8 Yeast lipases 19
    1.8.1 Introduction 19
    1.8.2 Characterisation of yeast lipase 20
    1.8.3 Mutation, selection and screening studies 20
1.9 Biotechnological applications 20
    1.9.1 Esterification 21
    1.9.2 Interesterification 22
    1.9.3 Biocatalytic resolution of optical isomers 23
    1.9.4 Polymer synthesis 23
    1.9.5 Intramolecular esterification 23
    1.9.6 Flavour development in food 23
1.10 Present and future applications of lipases 24
    1.10.1 Fat splitting 24
    1.10.2 Modification of oils and fats 25
    1.10.3 Synthesis of organic compounds 25
    1.10.4 Detergent products 25
1.11 Industrial applications of lipases 26
    1.11.1 Thermostable enzymes 26
    1.11.2 Usefulness of organic solvents 26
    1.11.3 Immobilisation 27
    1.11.4 Genetic engineering 28
    1.11.5 Oil and fat industry 28
    1.11.6 Dairy and food industry 29
    1.11.7 Miscellaneous 30

**CHAPTER 2 INTRODUCTION TO THE PRESENT STUDY 32**

**CHAPTER 3 MATERIALS AND METHODS 34**

3.1 Materials 34
    3.1.1 Analytical chemicals and resins 34
    3.1.2 Microorganism 34
3.2 Methods 34

3.2.1 Screening for lipase production 34
3.2.1.1 Lipase production on agar plates 35
3.2.1.2 Lipase production in liquid culture media 35
3.2.1.3 Optimisation of lipase production 36
3.2.2 Enzyme assays and protein determination 37
3.2.2.1 *p*NPP assay 37
3.2.2.2 Copper olive oil assay 38
3.2.2.3 pH-stat method 40
3.2.2.4 Protein determination 40
3.2.3 Electrophoresis 41
3.2.3.1 SDS-PAGE 41
3.2.4 Development of purification protocol 42
3.2.4.1 Purification of *Exophiala dermatiditis* UOFS Y-2044 Lipase (ED2044L) 42
3.2.4.1.1 Assessment of binding to MIMETIC A6XL dye adsorbent ligands using the Piksi kit® 42
3.2.4.1.2 First isolation attempt 43
3.2.4.1.3 Second isolation attempt 44
3.2.4.1.4 Third isolation attempt 44
3.2.4.1.5 Fourth isolation attempt 45
3.2.4.1.6 Fifth isolation attempt 45
3.2.4.1.7 Sixth isolation attempt 45
3.2.4.1.8 Seventh isolation attempt 46
3.2.4.2 Purification of *Exophiala dermatiditis* UOFS Y-2048 lipase (ED2048L) 47
3.2.4.2.1 Assessment of binding to MIMETIC A6XL dye adsorbent ligands 47
3.2.4.2.2 Purification protocol 47
3.2.5 Physical-chemical characterisation 48
3.2.5.1 Thermostability 49
3.2.5.2 Optimum temperature 49
3.2.5.3 Optimum pH 49
3.2.5.4 pH stability 49
3.2.5.5 Substrate specificity 50

3.2.5.6 Substrate concentration dependence 50

3.2.5.7 Effect of detergents 51

3.2.5.8 Effect of metal ions 51

3.2.5.9 Effect of EDTA 51

3.2.5.10 Effect of PMSF 52

**CHAPTER 4 RESULTS AND DISCUSSION 53**

4.1 Screening for lipase production 53

4.1.1 Lipase production on agar plates 53

4.1.2 Lipase production in liquid culture media 56

4.1.3 Optimisation of lipase production 57

4.2 Purification of *Exophiala dermatiditis* UOFS Y-2044 lipase 60

4.2.1 Assessment of binding to MIMETIC A6XL dye adsorbent ligands using the Piksi kit 60

4.2.2 First isolation attempt 63

4.2.3 Second isolation attempt 63

4.2.4 Third isolation attempt 64

4.2.5 Fourth isolation attempt 65

4.2.6 Fifth isolation attempt 65

4.2.7 Sixth isolation attempt 65

4.2.8 Seventh isolation attempt 66

4.3 Purification of *Exophiala dermatiditis* UOFS Y-2048 lipase 67

4.3.1 Assessment of binding to MIMETIC A6XL dye adsorbent ligands 67

4.3.2 Purification protocol 68

4.4 Characterisation of lipases 70

4.4.1 Molecular mass determination 70

4.4.2 Thermostability 71

4.4.3 Optimum temperature 72

4.4.4 Optimum pH 73

4.4.5 pH stability 73

4.4.6 Substrate specificity 74

4.4.7 Interfacial activation 75

4.4.8 Effect of detergents 76
4.4.9 Effect of metal ions 78
4.4.10 Effect of EDTA on lipase activity 80
4.4.11 Effect of PMSF on lipase activity 80

**CHAPTER 5 GENERAL DISCUSSION AND CONCLUSIONS** **82**

**REFERENCES** **84**

**SUMMARY** **93**

**OPSOMMING** **95**

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AG | acylglycerol |
| BCA | Bicinchoninic acid |
| BSA | Bovine serum albumin |
| $CaCl_2$ | Calcium chloride |
| CHAPS | 3-[(3-Cholamidopropyl)-dimethylammonio]-1 propane |
| cmc | critical micelle concentration |
| DAG | diacylglycerol |
| DEAE | Diethylaminoethyl |
| EDTA | Ethylene diaminetetraacetic acid |
| FA | fatty acid |
| HCl | Hydrochloric acid |
| IEC | Ion exchange chromatography |
| KCl | Potassium chloride |
| $M_r$ | Relative molecular mass |
| NaCl | Sodium chloride |
| NaOH | Sodium hydroxide |
| PMSF | Phenylmethylsulfonyl fluoride |
| *p*NP | *p*-Nitrophenyl |
| *p*NPP | *p*-Nitrophenylpalmitate |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| SEC | Size exclusion chromatography |
| TAG | Triacylglycerol |
| TRIS | 2-Amino-2-(hydroxymethyl)-1,3-propandiol |
| Tween-80 | Polyoxyethylenesorbitan monooleate |

# LIST OF FIGURES

Figure 1.1(a) Enzymatic reaction of a lipase catalysing hydrolysis/synthesis of a triacylglycerol substrate (Jaeger *et al.*, 1994)
1

Figure 1.1(b) Diagram of the enzymatic reaction of a lipase catalysing hydrolysis/synthesis of a triacylglycerol substrate (Jaeger *et al.*, 1994).
1

Figure 1.2 The interfacial activation of lipase. Diagrammatic representation of the conformational change in RmL (Taken from Brzozowski *et al.*, 1991).
5

Figure 1.3 Interfacial activation of lipases. (A) Classical activity profile of a pancreatic lipase and horse liver esterase at different substrate concentrations exceeding the saturation point. (B) Activity of *P.aeruginosa* lipase at different concentrations of triacetin (o, saturation concentration 306mM) and tripropionin (•, saturation concentration 15mM). (Taken from Jaeger *et al.*, 1994)
6

Figure 1.4 Three-dimensional structure of *Rhizomucor miehei* lipase showing the open and closed structures. Pictures were generated using Hyperchem software and co-ordinates obtained from the Brookhaven Protein Data Bank. (Taken from Jaeger *et al.*, 1994).
7

Figure 1.5 The catalytic mechanism of lipase. (Taken from Jeager *et al.*, 1994).
9

Figure 1.6 Model for description of interfacial kinetics with a water-soluble lipase enzyme acting on insoluble substrate. (Taken from Jeager *et al.*, 1994).
10

Figure 1.7 Variation in shape of an oil drop with time resulting from the action of a purified pig pancreatic lipase. (Taken from Labourdenne *et al.*, 1994).
16

Figure 1.8 Lipase kinetics, showing variations with time of interfacial tension, drop area and drop volume. The arrow indicates the beginning of the interfacial tension regulation. (Taken from Labourdenne *et al.*, 1994).
17

Figure 1.9 Industrially important reactions catalysed by a lipase. Transesterification involves the transfer of an acyl group to an alcohol

(alcoholysis) or glycerol (glycerolysis); interesterification describes the transfer of an acyl group to a fatty acid (acidolysis) or a fatty acid ester. (Taken from Jeager *et al.*, 1994).

22

Figure 3.1 The structure of *p*-nitrophenyl palmitate

37

Figure 3.2: Standard curve for assay of fatty acids released with the olive oil assay using stearic acid as standard. Standard deviations for the triplicate readings are shown.

39

Figure 3.3 Standard curve for BCA protein assay with BSA as protein standard. Standard deviations of triplicate readings are shown.

41

Figure 3.4 Standard curve for the Micro BCA protein assay with BSA as protein standard. Standard deviations of triplicate readings are shown.

41

Figure 4.1 Photographs showing a lipase-positive isolate growing on agar plates containing (i) Rhodamine B/ olive oil, (ii) Tween-80/ $CaCl_2$ and (iii) Tributyrin.

54

Figure 4.2 Time-dependent lipase production on different carbon sources monitored with the *p*NPP assay. (a) Profile of ED2044L and (b) profile of ED2048L.

56

Figure 4.3 Time-dependent lipase production after cells were grown to their maximum before induction.

57

Figure 4.4 Time-dependent lipase production of a young culture compared with an older culture. (a) Profile for ED2044L, (b) profile for ED2048L.

58

Figure 4.5 Time-dependent lipase production in water and olive oil medium.

59

Figure 4.6 Time-dependent lipase production when inoculating using a whole plate compared to inoculating using a loop. (a) a whole plate and (b) using a loop.

60

Figure 4.7 Elution profile of SEC on Toyopearl HW50F (0,1M TRIS-HCl buffer, pH8,5).

61

Figure 4.8 Elution profile of SEC on Toyopearl HW50F (0,025M phosphate buffer, pH6,0). 62

Figure 4.9 Elution profile of SEC on Toyopearl HW50F (0,01M phosphate buffer, pH5,8). 63

Figure 4.10 Elution profile of affinity chromatography on MIMETIC Yellow-1 A6XL adsorbent ligand (0,01M phosphate buffer, pH5,8). The arrow indicates the start of the gradient. 63

Figure 4.11 Elution profile of affinity chromatography on MIMETIC Red-2 A6XL adsorbent ligand (0,01M phosphate buffer, pH5,8). The arrow indicates the start of the KCl- gradient. 64

Figure 4.12 Elution profile of ion exchange chromatography on Toyopearl DEAE-650M (0,01M TRIS-HCl buffer, pH8,0). The arrow indicates the start of the KCl-gradient. 64

Figure 4.13 Elution profile of IEC on Toyopearl SP-650M column (0,05M phosphate buffer, pH5,8). 65

Figure 4.14 Elution profile of IEC on Toyopearl Super-Q 650S (0,05M phosphate buffer, pH8,0). The arrow indicates the start of the KCl-gradient. 66

Figure 4.15 Elution profile of SEC on Toyopearl HW50F (0,01M phosphate buffer, pH5,8). 67

Figure 4.16 Elution profile of affinity chromatography on MIMETIC Yellow-1 A6XL adsorbent ligand (0,01M phosphate buffer, pH5,8). The arrow indicates the start of the salt gradient. 67

Figure 4.17 Elution profile of affinity chromatography on MIMETIC Red-2 A6XL adsorbent ligand (0,01M phosphate buffer, pH5,8). The arrow indicates the start of the salt gradient. 68

Figure 4.18 Elution profile of IEC on Toyopearl SP-650M (0,05M phosphate buffer, pH5,8). 69

Figure 4.19 Elution profile of IEC on Toyopearl Super-Q 650S (0,05M phosphate buffer, pH8,0). The arrow indicates the start of the salt gradient. 69

Figure 4.20 SDS-PAGE gel of purified ED2044L. Lane 1 contains the marker proteins (listed in section 3.2.3.1); Lane 2 shows the isolated lipase. 70

Figure 4.21 Calibration curve of Rf-values vs. Log ($M_r$) used to determine the approximate $M_r$ of ED2044L. 70

Figure 4.22 Thermostability of ED2044L at different temperatures. Standard deviations are shown as error bars (n=3). 71

Figure 4.23 Thermostability of ED2048L at different temperatures. Standard deviations are shown as error bars (n=3). 72

Figure 4.24 Optimum temperature of ED2044L and ED2048L. Standard deviations are shown as error bars (n=3). 72

Figure 4.25 Optimum pH of ED2044L and ED2048L. Standard deviations are shown as error bars (n=3). 73

Figure 4.26 pH stability of ED2044L and ED2048L, after incubation at 37°C for 30 minutes. Standard deviations are shown as error bars (n=3). 74

Figure 4.27 Substrate specificity of ED2044L and ED2048L with (a) *p*-nitrophenyl esters and (b) triacylglycerols. Standard deviations are shown as error bars (n=3). 75

Figure 4.28 Interfacial activation of ED2044L and ED2048L with tripropionin as substrate. Standard deviations are shown as error bars (n=3). 76

Figure 4.29 Effect of detergent on ED2044L activity. Standard deviations are shown as error bars (n=3). 77

Figure 4.30 Effect of metal ions on ED2044L activity. Standard deviations are shown as error bars. 79

Figure 4.31 Effect of EDTA on lipase activity. Standard deviations are shown as error bars. 80

Figure 4.32 Effect of PMSF on ED2044L activity. Standard deviations are shown as error bars. 81

# LIST OF TABLES

Table 1.1 Assays for determination of lipase activity (Taken from Jaeger *et al.*, 1994). 11

Table1.2 Microbial lipases used as additives in household detergents. (Taken from Jaeger *et al.*, 1994) 21

Table 1.3 Biotechnological applications of bacterial lipases. (Taken from Jaeger *et al.*, 1994) 24

Table 3.1 Inducers used in growth media. 36

Table 3.2 Preparation of assay solution with varying concentrations of tripropionin. 51

Table 4.1 Screening results of 23 black yeast isolates on agar plates containing: (i) Tween-80/$CaCl_2$, (ii) Rhodamine B/ olive oil and (iii) Tributyrin. (*) Indicates low lipase production, (**) indicates medium lipase production and (***) indicates very high lipase production. 55

Table 4.2 Yields of ED2044L bound and not bound to MIMETIC A6XL adsorbent ligands (0,1M TRIS-HCl, pH8,5). 0,414Units applied to each 1ml column. 61

Table 4.3 Yields of EF2044L bound and not bound to MIMETIC A6XL adsorbent ligands (0,025M phosphate buffer, pH6,0). 0,3372Units were applied to each 1ml column. 62

Table 4.4 Purification table of ED2044L. 66b

Table 4.5 Purification table of ED2048L. 69b

Table 4.6 Thermostability of ED2044L and ED2048L. 72

# CHAPTER 1

# LITERATURE REVIEW

## 1.1 Introduction

Acylglycerol hydrolases (EC 3.1.1.3), or lipases, are enzymes that catalyse the reversible hydrolysis of tri-, di- or monoacylglycerols. Lipases can also catalyse the hydrolysis of a variety of compounds containing carboxylic ester moieties which are not acylglycerols (Jensen *et al.*, 1990). Although lipases can act on soluble monomeric substrates, practical utilisation of lipase-catalysed reactions is restricted to situations where the overall substrate concentration is higher than its solubility in the natural solvent, water. The property gives a way to differentiate lipases from conventional esterases, which ordinarily act on soluble monomeric substrates (Desnuelle, 1961). Lipases are a versatile class of enzymes, with the ability to catalyse the hydrolysis and synthesis of ester bonds in triacylglycerols. This ability was first recognised 71 years ago by Van der Walle in 1927 (Jaeger *et al.*, 1994). Lipases act on the carboxyl ester bonds present in triacylglycerols to liberate a mixture of fatty acids, glycerol and acylglycerols (mono-/di-). Their major substrates are long chain triacylglycerols (Figure 1.1), but some lipases can hydrolyse short chain acylglycerols. Lipases are widely distributed in all types of living organisms and are found in mammals, plants, fungi, bacteria and the archaebacteria (Olson *et al.*, 1994).

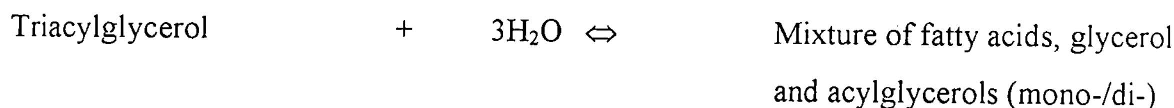



**Figure 1.1(a): Enzymatic reaction of a lipase catalysing hydrolysis/synthesis of a triacylglycerol substrate (Jaeger *et al.*, 1994)**

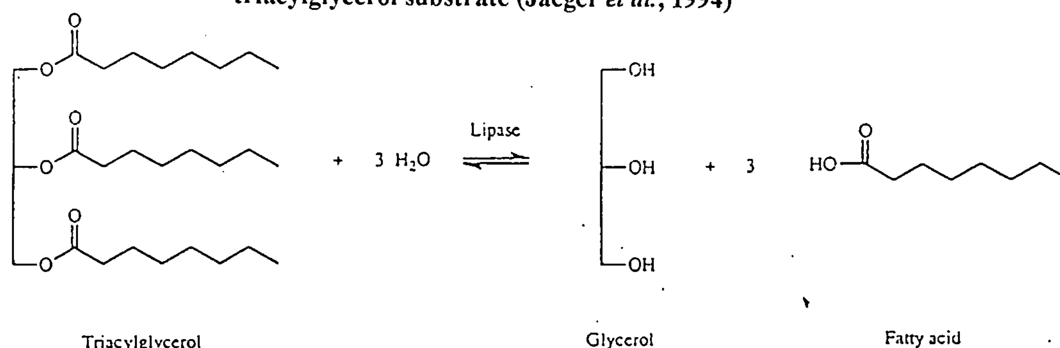



**Figure 1.1(b) : Diagram of the enzymatic reaction of a lipase catalysing hydrolysis/synthesis of a triacylglycerol substrate (Jaeger *et al.*, 1994).**

This literature review focuses on lipases in general and the differences between lipases produced by bacteria, yeasts and fungi. The presence of lipases in bacteria (*Bacillus prodigiosus*, *B. pyocyaneus* and *B. fluorescens)* had been observed as early as in 1901 by Eijkman (Jaeger *et al.*, 1994).

## 1.2 Substrate specificity

In general, lipases can be classified into four groups according to their substrate specificity.

### 1.2.1 Lipid class specificity

Lipid class specificity has been observed in animal plasma, which apparently contains separate lipoprotein lipases for the hydrolysis of triacylglycerols, diacylglycerols and monoacylglycerols (Jensen *et al.*, 1990). A lipase produced by a strain of *Penicllium cyclopium* (Okumura *et al.*, 1980) has been shown to display its highest activity on monoacylglycerols and much lower activities toward di- and triacylglycerols. This type of selectivity is dependent on temperature for a lipase from *Pseudomonas fluorescens* (McNeill *et al.*, 1990).

### 1.2.2 Positional specificity

Lipases obtained from natural sources can be positional non-specific or display one of two kinds of positional specificity : sn-1,3 specific or sn-2 specific (Kresse *et al.*, 1991). Non-specific lipases hydrolyse all three ester bonds of triacylglycerols equally well.
Examples are the lipases from

- *Chromobacterium viscosum* (Suguira and Isobe,1975)
- *Pseudomonas fluorescens* (Suguira *et al.*, 1977)
- *Candida cylindracea* (Benzonana and Esposito, 1971)
- *Geotrichum candidum* (Suguira *et al.*, 1977)
- *Penicilium cyclopium* (Okumura *et al.*, 1976)

Specificity of the sn-1,3 type is associated with the preferential release of fatty acid residues from the terminal positions of the glycerol backbone rather than from the central carbon atom, whereas sn-2 specificity refers to preferential release from the central carbon atom (Desnuelle, 1961).

### 1.2.3 Fatty acid specificity

Lipases often have a particular ability to release fatty acids whose chain lengths or degrees of unsaturation fall within well-defined regions. This situation has been explored in the lipase-catalysed production of flavours (Lindsay, 1985). For example, lipase from *Geotrichum candidum* shows specificity for the fatty acids with a double bond between C9 and C10 (Charton and Macrae, 1991). A bacterial lipase belonging to this group has yet to be found.

### 1.2.4 Stereospecificity

A number of researchers have observed stereoselectivity for the catalytic action of lipase on substrates such as straight-chain secondary alcohols, acetonide, butyric acid optically active esters, cyclohexanols, 2-benzylglycerol ether, sugar alcohols and several esters of ibuprofen (McConville *et al.*, 1990).

Stereospecificity of lipases is strictly dependent on the surface pressure of the substrate (Rogalska *et al.*, 1993; Ransac *et al.*, 1990a). An increase in the lipid density at the air-water interface decreased the stereo specificity of several lipases. Stereospecificity may also depend on the fatty acid chain length of the substrate.

## 1.3 Interfacial activation

Lipases are hydrolytic enzymes, which break down triacylglycerols into free fatty acids and glycerols. They have been classified as serine hydrolases owing to their inhibition by diethyl *p*-nitrophenyl phosphate. Lipase activity is greatly increased at the lipid-water interface, a phenomenon known as interfacial activation. X-ray analysis has revealed the atomic structures of two triacylglycerol lipases, unrelated in sequence, namely the human pancreatic lipase (hpl) and an enzyme isolated from the fungi *Rhizomucor miehei* (RmL). In both enzymes the active centres contain structurally analogous Asp-His-Ser triads (characteristic of serine proteases), which are buried completely beneath a short helical segment, or 'lid'. A complex of *R. miehei* lipase with *n*-hexylphosphate ethyl ester led to exposure of the active site by movement of the surface surrounding the catalytic site (Brzozowski *et al.*, 1991). Many factors have been suggested to trigger the activation

of lipases at a lipid-water interface. They include the increase of substrate concentration at the interface, better orientation of the scissile ester bond, reduction in the water shell around the estermolecules in water and a conformational change of the enzyme (Brzozowski *et al.*, 1991). A conformational change of the *R.miehei* lipase causes the helical lid to move away about seven Δ from the active site exposed to the environment. In the hypothesis of the activation the lid is stabilised in the open conformer by a hydrophobic environment, while it is closed in water (Norin *et al.*, 1993).

The necessary rearrangement of the enzyme is supported by the structures of three lipases reported (*Rhizomucor miehei* lipase, *Candida rugosa* lipase and *Goetrichum candidum* lipase). In all three of the lipases, the Ser-His-Asp/Glu catalytic triads are occluded by a polypeptide flap (lid) and are not exposed to the solvent (Grochulski *et al.*, 1993).

The conformational changes of the lid can be described as a simple rigid body movement of its helical part (residues Leu85-Asp91). It consists of a translation of the center of gravity of about eight Δ and a rotation of $167^0$ about an axis almost parallel to that of the helix (Figure 1.2a,b). There are two clearly defined hinge regions, the serine tripeptide 82-84 and the tetrapeptide 92-95, one on each side of the lid, which allows this movement. As the helical lid rolls back from the active site, its hydrophilic side, which is exposed to the solvent in the native structure, becomes partly buried in a polar cavity previously filled by well-ordered water molecules.

At the same time the hydrophobic side of the lid becomes completely exposed, greatly expanding the non-polar surface around the active site (Figure 1.2c,d). Thus the exposure of the catalytic residues is accompanied by a marked increase in the nonpolarity of the surrounding surface. Interfacial activation is thus explained by the stabilisation of this non-polar surface by the lipid environment which would in effect create a catalytically competent enzyme, able to attack the triacylglycerols (TAG) molecules within the lipid phase (Brzozowski *et al.*, 1991).

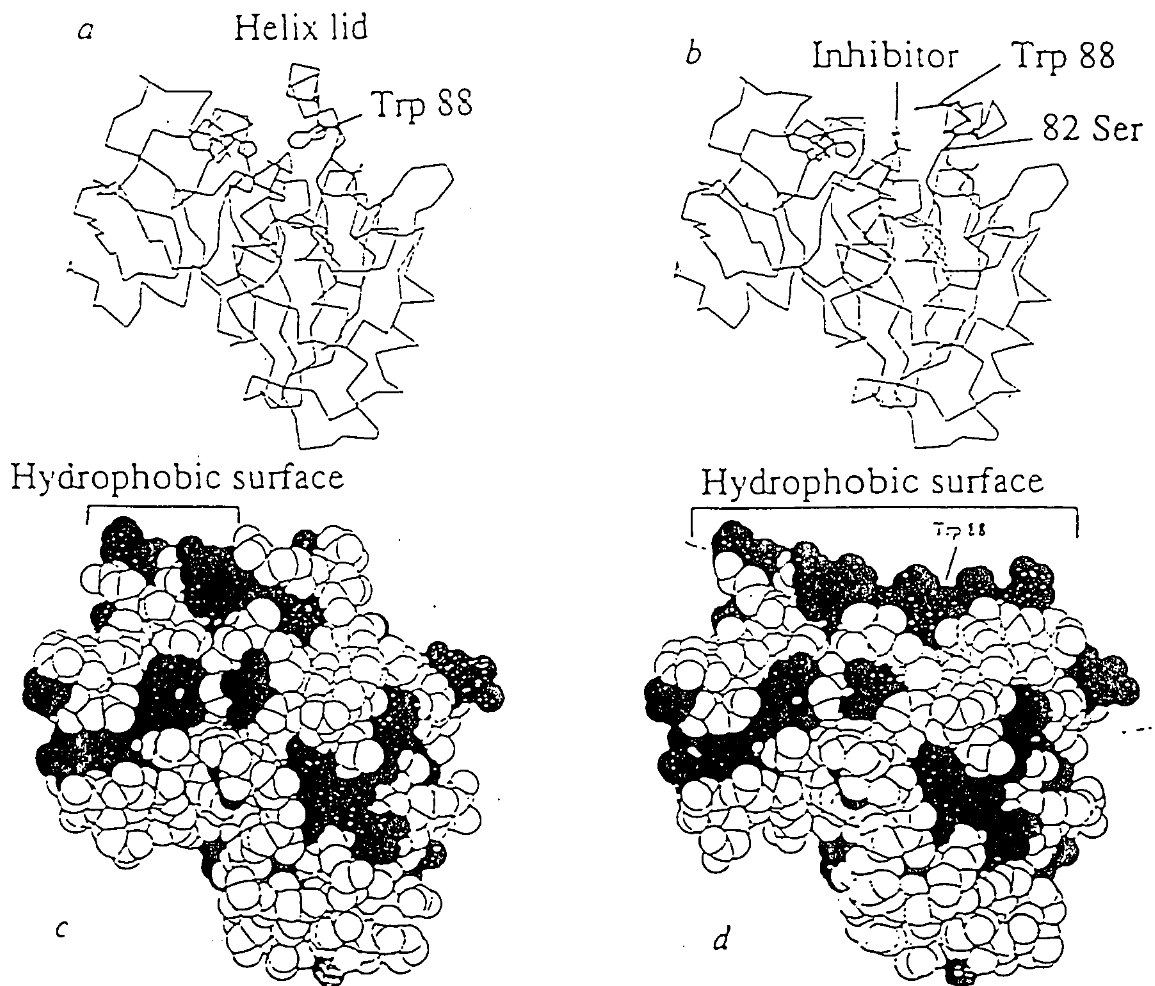



**Figure 1.2:** **The interfacial activation of lipase. Diagrammatic representation of the conformational change in RmL (Taken from Brzozowski *et al.*, 1991).**

Interfacial activation is the phenomenon where a sharp increase in lipase activity is observed when the substrate starts to form an emulsion, thereby presenting to the enzyme an interfacial area (Jaeger *et al.*, 1994). In contrast to esterases, lipases show very low activity with substrate as long as it is in its monomeric state (Figure 1.3), but when the solubility level of the substrate is exceeded, a sharp increase in enzyme activity is observed as the substrate starts to form a second phase (Sarda and Desnuelle 1958, 1961).

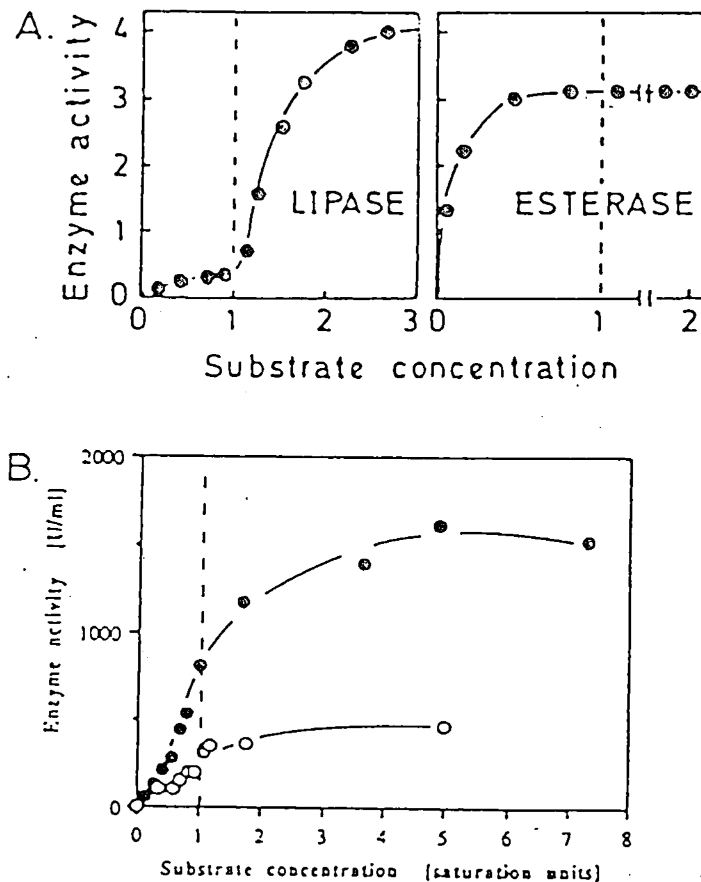



**Figure 1.3:** **Interfacial activation of lipases. (A) Classical activity profile of a pancreatic lipase and horse liver esterase at different substrate concentrations exceeding the saturation point. (B) Activity of *P. aeruginosa* lipase at different concentrations of triacetin (o, saturation concentration 306mM) and tripropionin (•, saturation concentration 15 mM) (Taken from Jaeger *et al.*, 1994).**

The vertical broken line represents substrate saturation. To the left of this line the substrate is dissolved in water. To the right the substrate forms an emulsion with an increasing interfacial area.

The fact that the lipase activity depends on the presence of an interface led to the definition of lipases as carboxylesterases acting on emulsified substrates (Brzozowski *et al.*, 1991, Van Tilbeurgh *et al.*, 1993).

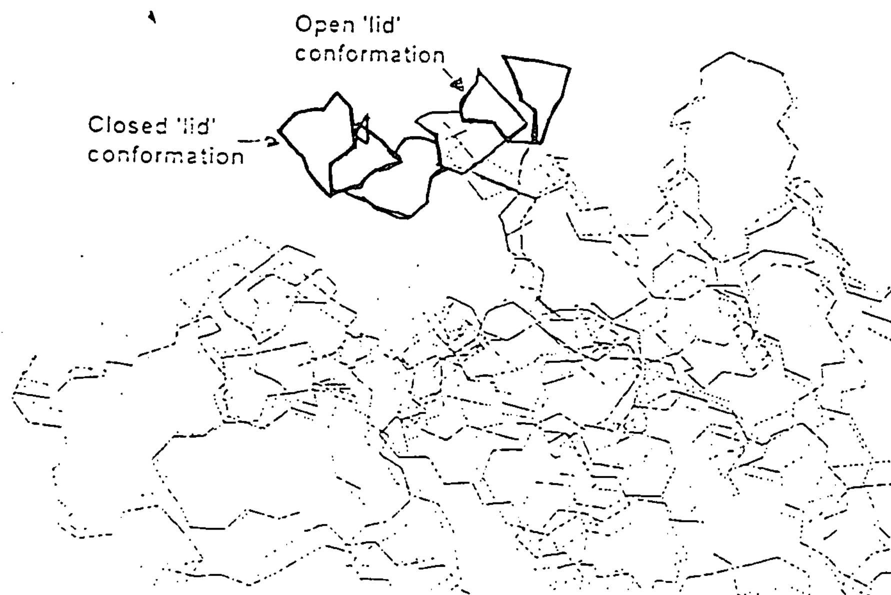



Figure 1.4: **Three-dimensional structure of *Rhizomucor miehei* lipase showing the open and closed structures. Pictures were generated using Hyperchem software and co-ordinates obtained from the Brookhaven Protein Data Bank (Taken from Jaeger *et al.*, 1994).**

These observations explained the phenomenon of interfacial activation with the lid causing inactivation if no lipid interface is present. This characteristic was then used to discriminate between "true lipases" and esterases, with "true lipases" being defined as enzymes which show interfacial activation in the presence of long chain triacylglycerols as substrates (Sarda and Desnuelle, 1958). Lipases should not be defined solely according to their interfacial activation behaviour, but also according to their capability to hydrolyse emulsions of long chain acylglycerols, the latter probably being the sole criterion distinguishing lipases from their esterase counterparts.

## 1.4 The catalytic site

The catalytic site of lipases is buried inside the protein and contains a serine-protease-like catalytic triad consisting of serine, histidine and aspartate or glutamate. The serine residue is located on the outside of a strictly conserved elbow structure, the $\beta$-$\varepsilon$-ser-$\alpha$ motif, which forces the serine to protrude away from the polypeptide chain, making it a good nucleophile. The active site is covered by a lid-like $\alpha$-helical structure, which moves away upon contact of the lipase with its substrate (Figure 1.4), thereby exposing hydrophobic residues at the protein's surface mediating the contact between protein and substrate. During the reaction a tetrahedral intermediate is formed which decomposes into an acyl-enzyme complex. The free lipase is regenerated by a hydrolytic reaction mediated by a water molecule (Figure 1.5). Firstly, a nucleophilic attack of the oxygen of the serine side chain on the carbonyl carbon atom of the ester bond leads to the formation of a tetrahedral intermediate (reaction 1). The histidine assists in increasing the nucleophilicity of the serine hydroxyl group. The histidine imidazole ring becomes protonated and positively charged which is stabilised by the negative charge of the acid residue (reaction 2). The tetrahedral intermediate is stabilised by two hydrogen bonds formed with amide bonds of residues which belong to the oxyanion hole. Finally, the alcohol first product is liberated leaving behind the acyl-enzyme intermediate (reaction 3). By nucleophilic attack of a hydroxyl ion (from water), the fatty acid is liberated and the enzyme regenerated (reaction 4) (Jaeger *et al.*, 1994).

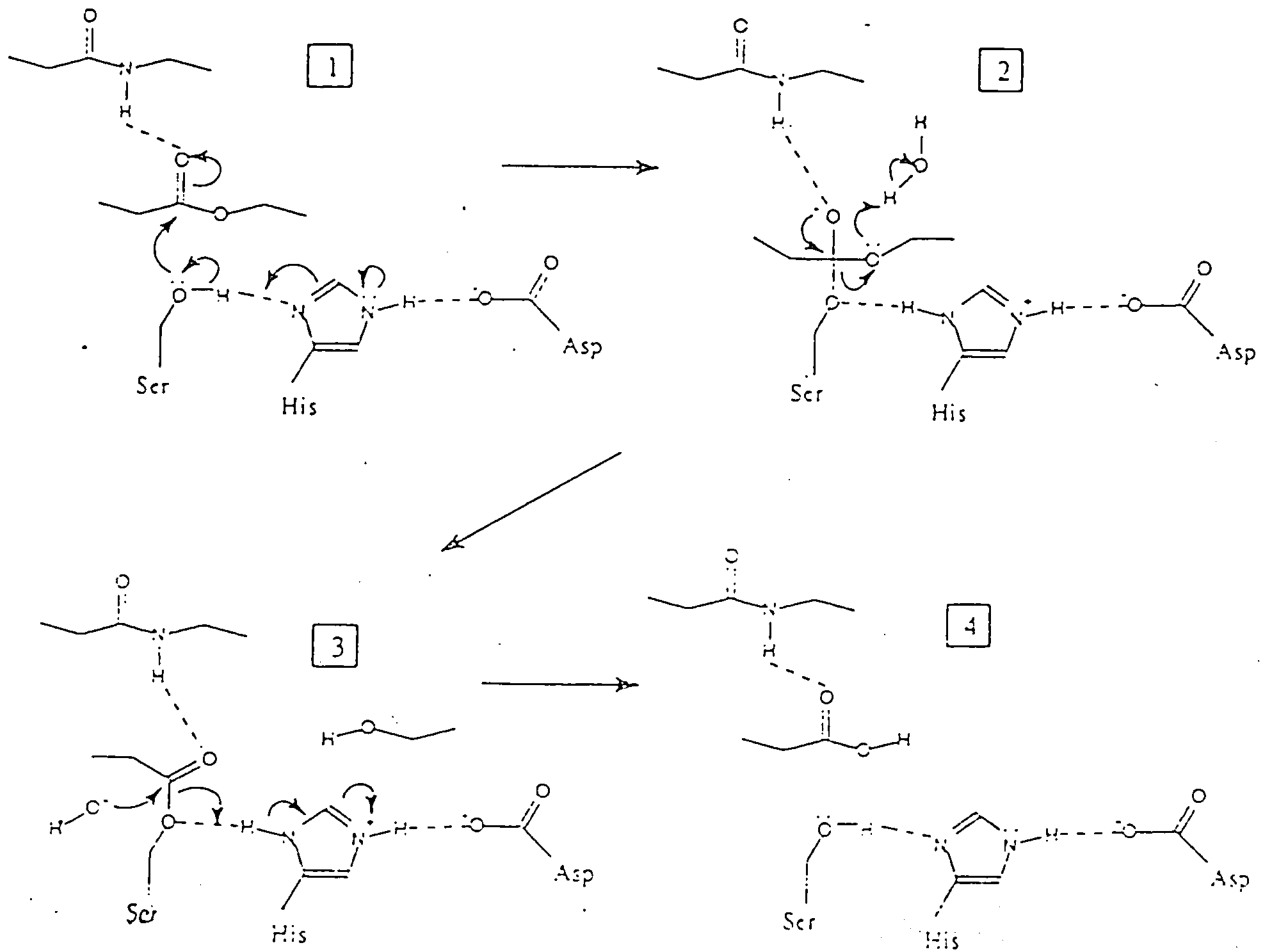



**Figure 1.5: The catalytic mechanism of lipase (Taken from Jaeger *et al.*, 1994).**

## 1.5 Kinetics of lipase

A lipase reaction does not follow the classical Michaelis-Menten (MM) model due to the presence of the interface and interfacial activation. The MM model is only valid in the case of one homogenous phase, i.e. for soluble enzymes and substrates. A model was proposed by Verger and De Haas in 1976 to describe the kinetics of catalysis by lipolytic enzymes (Figure 1.6). This model consists basically of two equilibrium steps :

1) The first describes the penetration of a water-soluble enzyme into an interface ($E \Leftrightarrow E^*$). It involves the physical adsorption of the enzyme at the lipid interface. This includes activation of the enzyme and the opening of the lid that blocks the active site (Brzozowski *et al.*, 1991; Van Tilbeurgh *et al.*, 1993).

2) The second equilibrium is one in which one molecule of penetrated enzyme binds a single-substrate molecule giving the complex E*S. Once the complex E*S is formed the catalytic step takes places, regenerating the enzyme in the form E* along with liberation of the products.

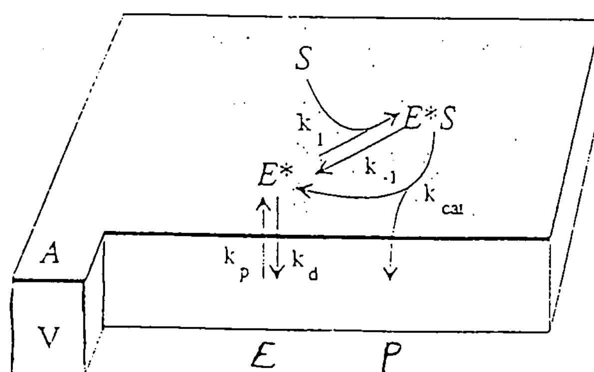



**Figure 1.6: Model for description of interfacial kinetics with a water-soluble lipase enzyme acting on insoluble substrate (Taken from Jaeger *et al.*, 1994).**

The second step may also be described by an "interfacial" Michaelis-Menten model with the substrate concentration expressed in moles/surface area rather than moles/volume. Equations have been derived which perfectly describe the experimental results (Verger and De Haas, 1976). The only case considered is one in which all the products of the reaction are soluble in the water phase,

diffuse rapidly and induce no change with time in the physicochemical properties of the interface. Additional models that describe the kinetics of competitive inhibition of lipases in the presence and absence of detergents, as well as for interfacial activation, have also been proposed (Ransac *et al*, 1990b;. 1991) .

## 1.6 Lipase activity determination

A number of lipase assay methods have been developed. Some of these are specific for the determination of mammalian lipase activity for diagnostic purposes. These assay procedures could at least partly be adapted for the determination of microbial lipase activity. A summary of currently used methods is given in Table 1.1 (Jaeger *et al.*, 1994).

**Table 1.1: Assays for determination of lipase activity (Taken from Jaeger *et al.*, 1994).**

| **Plate assays** | | |
|---|---|---|
| *Substrate* | *Reaction product* | *Method* |
| AG (triolein) | Free fatty acids | Coloured indicators |
| | | - Victoria blue |
| | | - Methyl red |
| | | - Phenol red |
| | | - Rhodamine B |

| **Spectroscopic** | | | | |
|---|---|---|---|---|
| *Substrate* | *Reaction product* | *Method* | *Final product* | *Wavelength* |
| 1.2 - DAG's | Glycerol | Enzymatic conversions | Quinone | 550nm |
| TAG's (triolein) | Free fatty acids | Enzymatic conversions | NAD | 340nm |
| AG's | Free fatty acids | Complex formation | Rhodamine 6G | 513nm |
| AG's (triolein) | Free fatty acids | Negative charge | Safranine | 520/560nm |
| AG's | Free fatty acids | Complex formation | Cu (II) salt | 715nm |
| *p*-nitro-phenyl | *p*-nitro-phenyl | Product is | | 410nm |

| esters | | coloured | | |
|---|---|---|---|---|
| 2.3-dimercapto-propan-1-ol tributyroate | Glycerol analogue | Reduction with DTNB | TNB | 412nm |
| Arylethene derivatives | Hydrolysis products are coloured | | | Variable |

| **Titrimetric** | | |
|---|---|---|
| *Substrate* | *Reaction product* | *Method* |
| TAG's (e.g. Tributyrin) | Free fatty acids | pH determination |

| **Fluorescence** | | | | |
|---|---|---|---|---|
| *Substrate* | *Reaction product* | *Method* | *Final product* | *Wavelength* |
| AG's (triolein) | Free fatty acid | Complex formation | 11(dansyl-amino) undecanoic acid | ex. 350nm em. 500nm |
| AG's Containing pyrene ring | Free fatty acid analogues or aggregated substrate | Fluorescense shift | Free fatty acid analogue or glyceride analogue | ex. 340nm em. 400nm 450nm |

| Surface pressure | | |
|---|---|---|
| *Substrate* | *Reaction product* | *Method* |
| Dicaprin | Free fatty acids | Measurement of barrier Movement |
| Long chain TAG's | Free fatty acids | Measurement of drop volume or decrease in surface tension |

### 1.6.1 Spectrophotometry

Several assays for lipase activity are based on spectroscopic measurements. Some of these assays make use of natural substrates yielding products that react with other compounds or may be used as substrates by other enzymes.

A number of examples are :

a) A colorimeter assay using long chain fatty acid 1,2-diacylglycerols. The lipase produces 2-monoacylglycerols from which glycerol is released. The glycerol concentration is determined by a sequence of enzymatic reactions with glycerol kinase, glycerol phosphate oxidase and a peroxidase that produce a violet Quinone monoimine dye with a peak absorbance at 550nm (Fossati *et al.*, 1992).

b) Rhodamine 6G used for complexation with free fatty acids liberated during lipolysis. A pink colour appears and adsorbance is monitored at 513nm (Jaeger *et al.*, 1994).

c) The metachromatic properties of safranine were used to detect a change in the net negative charge at the lipid-water interface, which was monitored by the change in absorbance of safranine. Very low amounts of enzyme can be detected (Rawyler and Siegenthaler, 1989).

d) Immobilised TAG's were hydrolysed and the released fatty acids were extracted with benzene and converted to the corresponding Cu(II) salts which were measured spectrophotometrically (Safarik, 1991).

e) Assays using substrate derivatives like β-naphtyl caprylate or 2,3-dimercaptopropan-1-ol tributyrate as substrate and 5,5'-dithiobis(2-nitrobenzoic acid) as chromogenic reagent (McKellar, 1986; Kurooka *et al.*, 1977).

f) Other substrates were substituted arylethene derivatives. The hydrolysis products of these compounds are coloured and many of them are water-soluble (Jaeger *et al.*, 1994).

g) Para-nitrophenyl-esters of various chain length fatty acids are also used as substrates (Stuer *et al.*, 1986).

Some of the spectrophotometric methods can be used in the presence of organic solvents which is useful when you are using the reverse micelle lipase purification technique.

### 1.6.2 Fluorescence

Traditional fluorimetric assays are discontinuous and like radiometric methods, require the separation of substrate from products. More recently, continuous fluorescence-based assays have been developed which rely on changes in the fluorescence properties of the substrate upon hydrolysis. In the future, chemiluminescent assays hold the promise of even higher sensitivity, but require the clever design of synthetic substrates (Hendrickson, 1994).

A number of fluorescent compounds have also been used for lipase assays :

a) In a continuous assay procedure the displacement of the fluorescent fatty acid probe 11-(dansylamino)undecanoic acid from a fatty acid binding protein was measured which is caused by long chain fatty acids released as a result of lipase activity (Jaeger *et al.*, 1994).

b) Non-fluorescent TAG's where one of the alkyl groups have been substituted with a fluorescent group such as a pyrenyl group can also be used. In an aggregated substrate the pyrene groups are closer together and fluoresce at 450 nm. When fatty acids are cleaved, the pyrene group's fluorescence shifts to 400 nm (Jaeger *et al.*, 1994).

### 1.6.3 Plate assays

Plate assays have been described for screening of lipase-producing microorganisms using either Victoria blue B, methyl red, Phenol red or Rhodamine B as indicators. Substrate hydrolysis causes the formation of colour of fluorescent halos around bacterial or yeast colonies. These methods are however, at best, qualitative or semi-qualitative (Samad *et al.*, 1989, Kouker and Jaeger, 1987).

### 1.6.4 Titrimetry

The lipolytic reaction liberates an acid, which can be titrimetrically assayed. A useful quantitative technique is to measure pH during the reaction course. The pH should be kept constant by continuously adding NaOH solution, the volume of which is monitored as a function of time. This method is called the pH stat method. The reaction rate obtained is a linear function of the lipase concentration and of the substrate concentration, the latter should be expressed in [moles/surface] area since the substrate is insoluble and forms an emulsion. Measurement should always be done under carefully controlled conditions to ensure reproducible quality of the interface (Jaeger *et al.*, 1994).

### 1.6.5 Controlled surface pressure

#### *1.6.5.1 The monolayer technique*

The effect of the surface pressure can be studied by the monolayer technique (Verger and De Haas, 1976). A monomolecular substrate film is spread at the air-water interface, which can be compressed with a surface barrier, changing the surface density of the substrate and thus the interfacial tension. The lipase injected into the water subphase will bind to the film and hydrolyse the substrate. The easiest way is to choose a substrate (e.g. trioctanoin, didecanoin or didodecanoin) which itself is insoluble in water, but which will generate soluble products. It is also possible to use substrates with longer acyl-chains under conditions where the surface pressure is above $23mM.m^{-1}$ and albumin is present in the subphase as a product-acceptor. When the substrate is hydrolysed, it will leave the interface, thereby decreasing the surface density and surface pressure which is then compensated by compression of the film by the mobile surface barrier. The barrier movement is monitored as a function of time. There are at least five major reasons for using lipid monolayers as substrates for lipolytic enzymes :

i) The monolayer technique is highly sensitive, and only small amounts of lipid are needed for kinetic measurements.

ii) During the course of the reaction, it is possible to measure several physicochemical parameters characteristic of the monolayer film, e.g. surface pressure, potential or radioactivity.

iii) The lipid packing in a monomolecular film of substrate is kept constant during the course of hydrolysis, and it is therefore possible to obtain a current presteady-state kinetic measurements with minimum perturbation caused by increasing amounts of reaction products.

iv) The "interfacial quality" can be modulated. It depends on the nature of the lipids forming the monolayer, their orientation and conformation, their molecular and charge densities, the water structure and the viscosity.

v) Inhibition of lipolytic enzyme activities by water-insoluble inhibitors can be precisely measured using a "zero-order" trough and mixed monomolecular film in the absence of any synthetic, non-physiological detergent. The monolayer technique is therefore suitable for modelling *in vivo* situations.

### *1.6.5.2 The oil drop method*

This method is based on the variations versus time in the oil/water interfacial tension from accumulation of water insoluble lipolytic production the surface of a triglyceride oil drop (Labourdenne *et al.*, 1994). This method consists of forming an oil drop in a water solution with the drop connected to a syringe containing the oil to be hydrolysed. The shape of the drop is directly correlated to the interfacial tension of the oil-water interface (Figure 1.7).

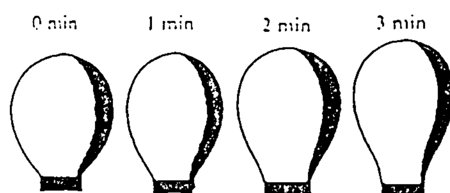



**Figure 1.7: Variation in shape of an oil drop with time resulting from the action of a purified pig pancreatic lipase (Taken from Labourdenne *et al.*, 1994).**

When the medium contains no detergent or fatty acid, the drop is shaped like an apple. When a lipase is added to the water phase, it binds to the oil-water interface and hydrolyses the substrate. The released products remain in the interface and the interfacial tension decreases. The shape of the oil drop now changes to a pear form and at a certain point it will leave the support. A computer-controlled device called an "oil-drop tensiometer" has been developed which automatically performs this type of lipase assay (Jaeger *et al.*, 1994).

The important features of the method and the apparatus for lipase assay are:

i) Linear response with enzyme concentrations ranging from 0.001 to 30 units/ml

ii) Independence

iii) By increasing the drop volume to maintain the interfacial tension constant, it is possible to monitor the enzyme kinetics and directly determine the number of molecules hydrolysed per unit time.

iv) Possibility of using natural long-chain triglycerides as substrates.

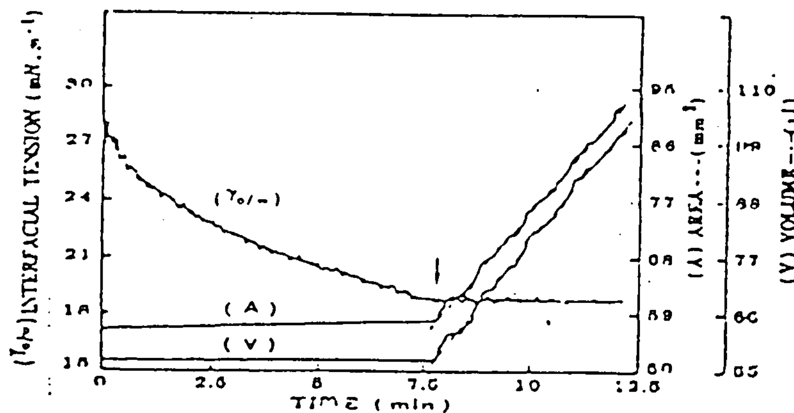



**Figure 1.8: Lipase kinetics, showing variations with time of interfacial tension, drop area and drop volume. The arrow indicates the beginning of the interfacial tension regulation. (Taken from Labourdenne *et al.*, 1994).**

### 1.6.6 Other assays

Some other assays to determine lipase activity include a high performance liquid chromatography assay involving the incubation of β-naphtyl laurate with enzyme followed by the quantification of naphtol after separating it from the assay solution by reverse phase HPLC (Jaeger *et al.*, 1994).

Another method uses NMR for quantitating lipase activity in biphasic macro emulsions or infrared spectroscopy for measuring lipase-catalysed hydrolysis of TAG in reverse micelles. Finally, a conductometric assay has been described using the short chain substrate triacetin (Jaeger *et al.*, 1994).

## 1.7 Purification procedures

Most of the purification procedures reported involve a series of non-specific techniques, such as ammonium sulphate precipitation, gel filtration, and ion-exchange chromatography. In recent years, affinity chromatography, reversed-micelle and aqueous two-phase systems, ultrafiltration membranes and immunopurification have also been applied to purify some lipases, mainly of microbial origin (Wooley and Petersen, 1994).

***Chromobacterium viscosum* lipase**

A potent bacterium for lipase production was isolated from soil and identified as *Chromobacterium viscosum*. The crude preparation contained more than two species of lipase, which differed from each other in molecular weight and isoelectric point (Suguira *et al.*, 1974). Lipase A was purified by chromatography using Amberlite CG-50 and Sephadex G-75. Lipase B was purified using Sephadex G-100, CM-cellulose and DEAE-Sephadex (Muderhwa *et al.*, 1985).

***Candida deformans* lipase**

*Candida deformans* CBS 2071 lipase was isolated and studied by Muderhwa *et al.* (1985). This enzyme was purified by acetone precipitation followed by chromatography on Sephadex C-50 and Sephadex G-150. The purification factor achieved was 70, and the protein and activity yields were 0.25% and 18% respectively.

The homogeneity of the purified enzyme was verified by polyacrylamide gel electrophoresis. The synthesis of this lipase is induced by lipid substrates in the culture medium and inhibited by glucose. This enzyme attacks primarily the 1-(or 3-) position of all triglycerides tested. Hydrolysis was preferential for triglycerides containing short chain fatty acids. The triglycerides with monounsaturated monoacids were more quickly hydrolysed than those with saturated monoacids. The presence of two and especially three double bonds in the fatty acid chain seemed to slow down the rate of hydrolysis (Muderhwa *et al.*, 1985).

***Aspergillus niger* lipase**

A lipase produced by *Aspergillus niger* was fractionated from the culture supernatant with ammonium sulphate (40-95%) and resuspended in 50mM potassium phosphate buffer (pH 6). Following filtration, the enzyme was introduced in a Pharmacia PD-10 column and eluted with 10mM potassium phosphate buffer (pH 6,0). The fraction containing the enzyme was collected and concentrated under vacuum (Hatzinikalaou *et al.*, 1996).

***Candida cylindracea* lipase**

10g of crude powder were suspended in 100ml of 25mM Tris-HCl buffer, stirred for 90 min and centrifuged. The supernatant was treated with ethanol and centrifuged again. The pellet was dissolved in buffer and dialysed over night. The solution was loaded on a DEAE-Sephacel column, developed and active fractions pooled and concentrated by ultrafiltration through Amicon PM30 membranes. The concentrated aliquots were loaded on a Sephacryl HR 100 column. This organism produced two lipases that were pure after the Sephacryl column step (Rùa *et al.*, 1993).

## 1.8 Yeast lipases

### 1.8.1 Introduction

Both academic and applied interests have stimulated the investigation of microbial lipases in recent yeast. The latter interest is due to the potential uses of lipases in, among others, digestive aids, hydrolysis of oils, interesterification of oils, flavour modification and esterification of fatty acids to glycerol, alcohols and carbohydrates. Although the ability to produce lipases is widely distributed

among microorganisms, the lipases of relatively few yeast species have been extensively studied (Finkelman, 1990).

### 1.8.2 Characterisation of yeast lipases

One of the best-studied yeast lipases is that of *Saccharomyces lipolytica*. The activity occurs as a cell-associated as well as an extracellular enzyme. The relative proportions are highly dependent on culture conditions and age. Grown in the absence of an inducer, the culture produces very little lipase. In the presence of olive oil or oleic acid, a large increase in the cell-associated form is observed (Finkelman, 1990; Rapp and Backhaus, 1992).

### 1.8.3 Mutation, selection and screening studies

This area appears to be relatively underdeveloped. One can find very little in the published literature dealing with either screening studies or strain development for increased output of yeast lipase. There are such studies in existence, but perhaps in closed, commercial files. This area appears ripe for exploitation using both the classical mutation and screening techniques and those of modern molecular biology. Many of the early techniques were reviewed by Finkelman (1990). In the main, the basis of these techniques is the formation of either zones of clearing in opaque media or colour change zones using dye-impregnated media. Kouker and Jaeger (1987) have demonstrated quantitation of lipase activity using fluorescent haloes, detected by ultraviolet irradiation of media containing lipid and Rhodamine B. Fluorescent haloes developed when lipolysis occurred. Use of Rhodamine B resolved problems encountered with the use of potentially bacteriostatic indicators such as Nile blue sulphate or Victoria blue and pH indicators (Finkelman, 1990; Jette and Ziomek, 1994).

## 1.9 Biotechnological applications

Lipases have the potential to catalyse both the hydrolysis and the synthesis of a variety of high-value industrial products, for example optically active compounds, various esters and lactones. More recently, lipases have been added to household detergents to reduce or replace synthetic chemicals that pose considerable environmental problems. For several decades the use of lipases in industry was rather small and the major applications were for flavour development in food such

as Italian cheeses (Jaeger *et al.*, 1994). The reasons for this limited application in industry were mainly due to the limited availability and relatively high costs of these enzymes. Furthermore, the lipases that were applied on industrial scale were of fungal or yeast origin, mainly due to their GRAS (generally regarded as safe) status. However, for various applications bacterial lipases are as good as or sometimes preferred to their eukaryotic counterparts (Table 1.2).

Table 1.2: **Microbial lipases used as additives in household detergents (Taken from Jaeger *et al.*, 1994).**

| Origin of lipase | Product name | Year of introduction | Company (location) |
|---|---|---|---|
| **Fungal** | | | |
| *Humolica lanuginosa* | Lipolase | 1988 | NOVO-Nordisk (Denmark) |
| **Bacterial** | | | |
| *Pseudomonas mendocina* | Lumafast | 1992 | Genencor (USA) |
| *Pseudomonas alcaligenes* | Lipomax | 1995 | Gist-brocades (Netherlands) |
| *Pseudomonas glumae* | n.a. | n.a. | Unilever (Netherlands) |
| *Pseudomonas species* | n.a. | n.a. | Solvay (Belgium) |
| *Bacillus pumilus* | n.a. | n.a. | Solvay (Belgium) |

n.a., no annotation

The lipase-catalysed reactions are mainly :

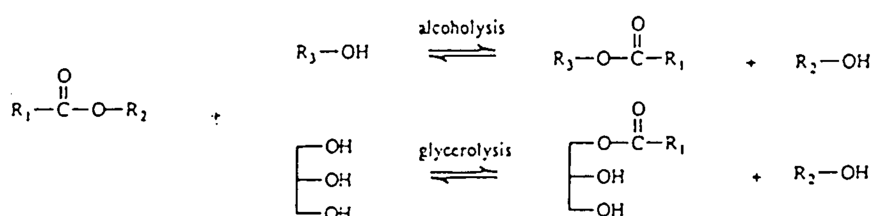
### 1.9.1 Esterification

Acylglycerols can be obtained by direct esterification of free fatty acids and glycerol. A process resulting in regio-isomerically pure acylglycerols has been developed comprising as an essential step the adsorption of glycerol onto a solid support (Berger *et al.*,1992; Berger and Schneider,1992). Lipase-catalysed acylglycerol synthesis with the immobilised glycerol and various acyl donors yielded multigram quantities of regio-isomerically pure di- and monoglycerols. *C. viscosum* was one of the 1,3-selective lipases producing the desired acylglycerols with high yield (Jaeger *et al.*, 1994).

## 1.9.2 Interesterification

A few lipase catalysed synthesis reactions in low-water environment have found limited application on commercial scale, e.g. the transformation of low-value oils, like the palm oil mid fraction, into high-value cocoabutter triacylglycerols by interesterification (Figure 1.9) (Babayan, 1987).

Transesterification




Interesterification

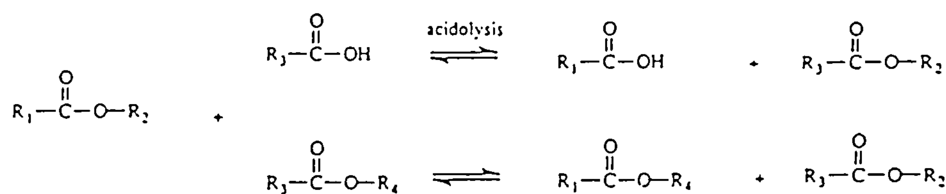



Figure 1.9: **Industrially important reactions catalysed by a lipase. Transesterification involves the transfer of an acyl group to an alcohol (alcoholysis) or glycerol (glycerolysis); interesterification describes the transfer of an acyl group to a fatty acid (acidolysis) or a fatty acid ester (Taken from Jaeger *et al.*, 1994).**

### 1.9.3 Biocatalytic resolution of optical isomers

By far the most important application of lipases in organic chemistry is the production of optically active compounds. Optically active building blocks for insecticides have been obtained by an ester hydrolysis reaction using Arthrobacter lipase (Mitsuda *et al.*, 1988).

### 1.9.4 Polymer synthesis

If a diester and a diol are used instead of a racemic ester and alcohol, stereoselective polycondensations occur in organic media. The formation of optically active trimers and pentamers in this way was observed, using, amongst others, a lipase from *Chromobacterium* species (Jaeger *et al.*, 1994).

### 1.9.5 Intramolecular esterification

If hydroxyl and acid moieties are present in one molecule, intramolecular esterification occurs, resulting in the synthesis of macrocyclic lactones. $C_{14}$-$C_{16}$ macrocylic lactones are high-grade and expensive substances with a musky fragrance, which are used in perfumes. In addition, microcyclic lactones can be synthesised by direct condensation of diacids with diols (Jaeger *et al.*, 1994).

### 1.9.6 Flavour development in food

Traditionally, bacterial lipases produced *in situ* in various food systems have been involved in development of flavour. Lipases from mainly *Pseudomonas* species present in raw milk are known to withstand the pasteurisation process and affect flavour development during cheese ripening. Other examples of the involvement of lipolytic lactic acid bacteria in flavour development are vegetable fermentations and ripening of some Italian sausages (Wooley and Petersen, 1994).

Table 1.3: **Biotechnological applications of bacterial lipases (Taken from Jaeger *et al.*, 1994)**

| Type of reaction | Origin of lipase | Product (application) |
|---|---|---|
| Hydrolysis of fat & oil | *Pseudomonas* | |
| Glycerolysis of fat & oil | *Pseudomonas* | Monoacylglycerols (surfactants) |
| Esterification to glycerol | *Chromobacterium viscosum*<br>*Pseudomonas Fluorescens* | |
| (Trans)esterification to immobilised glycerol | *Chromobacterium viscosum* | |
| Acylation of sugar alcohols | *Chromobacterium viscosum* | Sugar monoacylesters (surfactants)<br>Enrichment of PUFAs |
| Acidolysis/Alcoholysis of fish<br>Oils | *Pseudomonas* | |
| Resolution of racemic Alcohols/esters | *Arthrobacter*<br>*Pseudomonas cepacia* | Building blocks for Insecticides/chiral drugs |
| Polytransesterification of Diesters with diols | *Chromobacterium*<br>*Pseudomonas*<br><br>*Pseudomonas* | Oligomers<br>Alkyds (polyester Intermediates)<br>Macrocyclic lactones |
| Transesterification of Monosaccharides | *Pseudomonas (cepacia)* | Acrylate esters (polyacrylate Intermediates) |
| Intramolecular esterification | *Pseudomonas* | Macrocyclic lactones |

## 1.10 Present and future applications of lipases

### 1.10.1 Fat splitting

Oils and fats are hydrolysed industrially to produce free fatty acids, soaps and glycerol. Historically, castor-bean lipase has been used to split castor oil, but with the development of more

efficient chemical methods this enzyme-catalysed process fell into disuse. However, the availability of comparatively cheap microbial lipases has led to a renewed interest in the use of lipases as fat-splitting catalysts. Lipases with no regiospecificity are particularly suitable because they catalyse the complete hydrolysis of triacylglycerols to fatty acids and glycerols. For example, Linfield *et al.* (1984) have shown that *Candida rugosa* lipase can be used to give 95-98% hydrolysis of tallow, coconut oil and olive oil. The Myoshi Oil Company of Japan have reported operation of a process by this lipase for production of fatty acids to be used for soap production (Macrae and Hammond, 1985).

### 1.10.2 Modification of oils and fats

The hydrolysis of triacylglycerol by lipase is an equilibrium reaction, as seen in Figure 10 (Macrae and Hammond, 1985). The equilibrium may be perturbed by altering the concentrations of the reactants and/or products and it has proved possible to shift the equilibrium in the direction of ester synthesis. Exploitation of various aspects of lipase specificity allows the synthesis of compounds that are difficult to prepare by chemical routes. This application involves the interesterification reaction (Macrae and Hammond, 1985).

### 1.10.3 Synthesis of organic compounds

The broad substrate specificity of lipases has been employed in studies of synthesis of various compounds other than triacylglycerols. While a considerable range of compounds of diverse chemical structure may be acted upon, the enzymes retain regio- and/or stereospecificity, allowing the preparation of compounds difficult to obtain by chemical routes. The reactions catalysed may be hydrolyses, ester syntheses or ester-exchange reactions. Ester synthesis reactions make use of the law of mass action to drive the equilibrium in the direction of synthesis by removing water generated during the reaction (Macrae and Hammond, 1985).

### 1.10.4 Detergent products

A large potential market for lipolytic enzymes is in detergent formulations where they could be effective in removing fatty deposits, particularly at low washing temperatures. Lipases are unable to digest most fatty deposits to fully water-soluble products but their action may improve soil removal by the surfactants present in the formulation. Indeed, some improvement in detergent performance might well be evident as a result of improved surfactant properties of lipolysis

products themselves, as noted when phospholipase A-2 acts on lecithin (Starace, 1983). Detergent applications represent a potential high-tonnage use of lipases in the future (Macrae and Hammond, 1985).

Lipases show a potential use in washing powders and surfactants (Phillips and Pretorius, 1991). Derivatives made from fatty acids coming from the hydrolysis of oils and fats can be used in the production of soap, surfactant, plasticisers and lubricants. A continuous high-pressure hydrolysis process was used for the hydrolysis of fats and oils, but a new hydrolysis method using lipase was recently devised. The use of lipase reduced the requirement for energy and the hydrolysis can be conducted under mild conditions (Tanigaki *et al.*, 1993).

Lipases with 1.3-specificity such as the lipases of *Rhizomucor miehei* and *Humicola lanuginosa* (Lipolase™) are used in laundry detergents (Zamost *et al.*, 1991). Trans- and interesterification reactions carried out by several lipases can be used in the production of surfactant (Shabtai and Daya-Mishne, 1992).

## 1.11 Industrial applications of lipases

At present there is an increasing interest in the development of applications for the lipases, particularly in the detergents, oils and fats, pharmaceutical, dairy and food, pulp and paper industries and the immobilisation of enzymes (Sztajer *et al.*, 1992). A large number of lipases have been screened for application as food additives, industrial reagents, cleaners as well as for medical application e.g. digestive drugs and diagnostic enzymes (Taipa *et al.*, 1992).

Lipases catalyse a variety of biotechnologically relevant reactions e.g. the purification of free fatty acids, oils and fats and the synthesis of esters and peptides with a range of properties depending on their sources (Schmidt-Dannert *et al.*, 1994). The production of esters with desirable physical and chemical properties through the employment of hydrolysing enzymes such as lipases is of great importance and interest from an industrial viewpoint (Sztajer *et al.*, 1992).

The world market for industrial enzymes has been estimated at approximately US $600 million, with lipases comprising approximately US $20 million in 1993 (Gilbert, 1993). However, the search for potential industrial applications of bacterial, fungal and yeast lipases was broadened by the availability of large quantities of microbial lipases by bioprocesses. Successful application of

thermostable enzymes, lipase activity in organic solvents, immobilised enzymes, recombinant DNA technology and protein engineering has enabled lipases to effectively compete with other well-established chemical technologies (Taipa *et al.*, 1992).

### 1.11.1 Thermostable enzymes

There exist a few advantages in using thermostable enzymes in industrial processes in comparison to thermolabile enzymes. One of the main advantages is the increase in reaction rate as the temperature of the process is increased. An increase of 10°C in temperature approximately doubles the reaction rate, which in turn decreases the amount of enzyme needed. Thermostable enzymes are also able to tolerate higher temperatures, which lead to a longer half-life. The use of higher temperature in industrial enzyme processes may also be useful during mixing processes, causing a decrease in the viscosity of liquids and may allow higher concentrations of low solubility materials. The mass transfer rate is also increased at higher temperatures as in the rate of many chemical reactions. Another advantage of the use of higher temperature during industrial processes, is the inhibition of microbial growth, which decreases the possibility of microbial contamination (Zamost *et al.*, 1991).

According to Zamost *et al.* (1991) the use of thermostable enzymes from thermophilic organisms has been increasing recently due to the cloning of genes from thermophiles into mesophilic production strains.

### 1.11.2 Usefulness of lipases in organic solvents

In nearly anhydrous organic solvents lipases are able to catalyse reverse reactions of synthesis and group exchange of esters as well as the resolution of racemic mixtures into optically active alcohols or acids, especially when these compounds are unstable or poorly soluble in water. Several systems and classes of lipases have been successfully employed for synthetic purposes (Taipa *et al.*, 1992; Ottolina *et al.*, 1992). However, the natural activity of lipases is concerned with ability to perform selected acylation. Lipase regioselectivity has been shown to proceed with quite different properties in organic solvents than in water, in part due to the diminished rate of acyl group migration (Pedrocci-Fantoni and Servi, 1992).

The most frequently investigated systems using organic solvents are two-phase systems consisting of a solid enzyme, either in powder form or adsorbed onto a solid support, in suspension in an apolar solvent. Biphasic systems can also be used which consist of a water-immiscible organic

solvent and an aqueous phase containing enzyme in solution. An alternative to a two-phase system is a homogeneous system e.g. a reverse micellar solution (Borzeix *et al.*, 1992).

Application of lipases to polyhydroxylated substrates are numerous and refer mainly to the carbohydrate field. Different synthetic applications also exist e.g. in steroid and oligonucleotide chemistry and isolated cases of multifunctional substrates (Pedrocci-Fantoni and Servi, 1992).

### 1.11.3 Immobilisation

An alternative approach for the use of lipases in industries is immobilisation of enzymes on hydrophilic supports, due to the ease of reutilisation of the enzyme (Virto *et al.*, 1994). Lipase used for transesterification reactions, acidolysis and ester synthesis are immobilised to increase their thermostability and extended use in columns. Enzymes modified in this way are much more stable and have a higher optimal temperature which is ideal for industrial use. The optimal temperature of the lipase of *Rhizomucor miehei* increased from 40°C to about 70°C after immobilisation (Zamost *et al.*, 1991).

The various immobilisation methods can be subdivided into two main categories: physical methods and chemical methods. Physical methods make use of weaker interaction or mechanical containment of the enzyme. Immobilisation by chemical methods incorporates difficult chemistry and requires the use of expensive and sometimes toxic reagents (Zamost *et al.*, 1991).

Immobilisation by adsorption is simpler, less expensive and is known to retain high catalytic activity. A strong hydrophobic or electrostatic interaction is needed between the enzyme and the support for immobilisation by adsorption to be successful. Strong electrostatic interaction can be achieved by using highly charged supports or charged enzymes. Strong hydrophobic interactions can be achieved by using hydrophobic supports or hydrophilic enzymes (Zamost *et al.*, 1991).

Lipases have been used with success as immobilised enzymes. The hydrophobicity of lipases could be increased by attaching various hydrophobic groups onto the enzyme molecule. Lipases could be modified with monomethoxypolyethylene glycol (PEG), alkylated with acetaldehyde and dodecyldehyde or aminidated with acetonimidate and methyl 4-phenylbutyrimidate (Basri *et al.*, 1994).

Successful utilisation of immobilised enzymes could only be ensured by efficient contact between the stationary and the mobile phases during hydrolysis reactions. A membrane bioreactor system using hydrophilic and hydrophobic microporous membranes was found to be capable of separating the mobile phase e.g. the oil or fat and water efficiently recovering lipases all at the same time in a heterogeneous reaction. The hydrolysis of a large quantity of soybean oil was possible in long-term semi-continuous operation without adding fresh enzyme (Tanigaki *et al.*, 1993).

The lipase of *Bacillus thermocatenulatus* was found to bind almost irreversibly to resins such as Phenyl-Sepharose, Amberlite, Serolite and Q-Sepharose. These immobilised enzymes proved to be very stable. No loss of activity could be detected after storing lipase immobilised on Phenyl-Sepharose for one week at room temperature. Lipase immobilised on Amberlite was even stable after two months storage at room temperature (Schmidt-Dannert *et al.*, 1994).

### 1.11.4 Genetic engineering

Industrial lipases have been used in rapidly increasing scale for an expanding variety of processes over the last two to three decades. Enzymes intended for use on an industrial scale have to be produced at relatively low cost. To reach this goal high expression levels are necessary. This can be obtained through traditional mutagenesis or strain selection procedures or through molecular cloning or heterologous expression. An example is the high level expression of the *Humicola lanuginosa* lipase (Lipolase™) in an *Aspergillus oryzae* strain by NOVO NORDISK (Boel *et al.*, 1990).

### 1.11.5 Oil and fat industry

One of the most important markets for the use of lipases is digestive oils, for which microbial lipases replace pancreatic lipase, which is expensive and scarce.

In the field of oil and fat hydrolysis lipases are increasingly recognised as very versatile catalysts especially for interesterification reactions. Lipases can also be used as biochemical catalysts to restructure triacylglycerols present in fats and oils (Vora *et al.*, 1988). Advantages of lipases over conventional catalysts are the specificity of the enzyme-mediated reactions and the mild conditions (Derksen and Cuperus, 1992).

Palm oil is a solid at room temperature due to the large percentage of palmitic acid present. This oil can be converted into fluid oils by the substitution of its palmitic acids. This can be achieved by fermentation processes or by using lipases. The fluid fraction can be used for cooking and seasoning after refining and it has a much higher market value than the solid fraction (Muderhwa *et al.*, 1985).

### 1.11.6 Dairy and food industry

Interesting applications for microbial lipases to make food more palatable and acceptable in the dairy and food industries have been reported (Muderhwa *et al.*, 1985).

Lipases from organisms such as *Aspergillus niger, A. oryzae* and *Saccharomyces* species are used for dairy based flavouring preparations. Lipases can also be used for the flavouring, colouring and processing of cheese e.g. the lipases of *Penicillium camemberti* and *P. caseicolum* which are responsible for flavour development of Camembert and Brie cheeses, respectively (Alhir *et al.*, 1990).

Another use of lipases in the food industry is the catalysing of reactions such as the rearrangement of cheap vegetable oil to a cocao butter equivalent with considerable commercial value. Lipases are also being used for the large scale production of other modified triacylglycerols such as human milk fat replacers for use in processed dairy products (Bosley, 1994).

Talon *et al.*, (1993) reported that the combined use of lactic acid bacteria and staphylococci in meat curing has been an established feature in the meat technology of many countries. *Micrococcaceae* are used as starters due to their ability to reduce nitrate, produce catalyse and contribute to flavour through their lipolytic activities.

In some fats as in beef tallow, the fatty acids are not always in liquid form at normal enzyme reaction temperatures. Efficient enzymatic processes for these substances would require suitable thermostable lipases. These thermostable lipases can also be used in pasteurised foods which were heat treated with high temperature short time sterilising processes (Chung *et al.*, 1991).

### 1.11.7 Miscellaneous

Lipases are used in the production of cosmetics (Odera *et al.*, 1986) as well as to hydrolyse triacylglycerols in sulfite treated pulp resins in the paper industry.

Lipases have been used widely as chiral catalysts in transesterification, interesterification and esterification reactions for kinetic resolution and asymmetrisation of prochiral compounds. Usually only one of the components, either the alcohol or the acylation agent, is chiral or prochiral. Only a few lipase catalysed reactions of this type exist in which both partners are chiral (Theil and Björkling, 1993).

# CHAPTER 2

# INTRODUCTION TO THE PRESENT STUDY

The world market for industrial enzymes, especially lipases are considerable. The importance of these enzymes in biotechnology and synthetic chemistry is illustrated by the BRIDGE Lipase T Project which was established by the EEC in 1982. The aim of this program was the characterisation of new lipases with respect to their 3D structure, function, protein engineering and catalytic mechanisms, as well as the industrial application of lipases.

Microbial lipases are very diverse in their enzymatic properties and substrate specificities, which make them attractive for industrial applications. A large number of lipases have been screened for application as food additives, industrial reagents and as detergent additives, as well as for medical applications.

Most of the microbial lipases are extracellular, being excreted through the external membrane into the culture medium. Optimisation of fermentation conditions influence the properties of the enzyme produced as well as the concentration ratio of extracellular to intracellular lipases (Taipa *et al.*, 1992). The amount of enzyme produced is dependent on several environmental factors, such as cultivation temperature, pH, nitrogen composition, carbon and lipid sources, concentration of inorganic salts and availability of oxygen.

A wide range of fractionation methods and chromatographic media are implemented in the purification of lipases. For the purification of microbial lipases, anion exchange remains one of the most popular separation techniques. It has been used in the purification of lipases from a variety of sources, for example *Pseudomonas fragi* (Nishio *et al.*, 1987), *Staphylococcus hyicus* (Van Oort *et al.*, 1989) and a lipase from *Bacillus* species (Sugihara *et al.*, 1991; Nthangeni, 1997). Gel filtration was also used with great success in the purification of lipases of *Humicola lanuginosa* (Omar *et al.*, 1987), *Candida deformans* (Muderhwa *et al.*, 1985), *Chromobacterium viscosum* (Suguira *et al.*, 1974) and *Fusarium heterosporum* (Shimada *et al.*, 1993). The use of hydrophobic interaction chromatography in the purification of lipase has been reported for *Pseudomonas cepacia* (Dünhaupt *et al.*, 1992) and *Galactomyces geotrichum* (Phillips and Pretorius, 1991). The use of

hydroxylapatite chromatography in the purification has been reported for *Bacillus subtilis* 168 lipase (Lesuisse *et al.*, 1993).

For the present study, black yeast isolates were made available from the culture collection in the Department of Microbiology and Biochemistry, U.O.F.S.. Positive screening of the organisms on Rhodamine B/olive oil, Tween-20, Tween-80, Tributyrin and Glucose agar plates identified 23 suitable lipase producers. The objectives of the present study were to use one or two isolates in a study involving , in sequence : (i) optimisation of lipase production, (ii) purification of specific lipases (iii) characterisation of the lipases. Desirable properties would be high lipase activity, high temperature optimum and stability and the ability to function in extremes of pH.

Different combinations of chromatographic methods were used including: cation exchange chromatography (SP Toyopearl 650M), anion exchange chromatography (Toyopearl Super-Q 650S), gel filtration (Toyopearl HW50F) and affinity chromatography (MIMETIC Red and Yellow A6XL dye adsorbent ligands, depending on the organism worked with).

The following properties of the lipase were characterised : molecular mass, thermostability, optimum pH and temperature, substrate specificity, interfacial activation, the effect of PMSF, the effect of EDTA, the effect of detergents and the effect of metals.

# CHAPTER 3

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Analytical chemicals and resins

Analytical reagent grade chemicals were obtained from commercial sources and were used without further purification. Unless mentioned otherwise, all reagents were purchased from Sigma. The nutrient broth was purchased from Biolab Diagnostics and Holpro supplied the D-glucose. The following chromatography media were obtained from : (1) Tosohaas : Toyopearl DEAE 650M, Toyopearl Super-Q 650S, Toyopearl Phenyl 650M, Toyopearl HW50F and Toyopearl SP 650M and (2) Affinity Chromatography Ltd. : MIMETIC Red A6XL dye adsorbent ligand and Yellow A6XL adsorbent ligands. The ultrafiltration stirred cell and YM-10 membranes were from Amicon and the Minitan Ultrafiltration unit equipped with polysulphone membranes (molecular mass cut-off of 10 000) from Millipore. Phenylmethylsulphonyl fluoride (PMSF) and calibration proteins for SDS-PAGE were supplied by Boehringer Mannheim.

### 3.1.2 Microorganism

A total of 23 black yeast isolates were screened for lipase production. The isolates were made available from the culture collection in the Department of Microbiology and Biochemistry.

## 3.2 Methods

### 3.2.1 Screening for lipase production

### *3.2.1.1 Lipase production on agar plates*

Screening for lipase production was done by streaking black yeast isolates on agar plates containing four different inducers, namely : (i) olive oil/Rhodamine B, pH7,0 (Kouker and Jaeger, 1987); (ii) Tween-20/$CaCl_2$, pH7,0; (iii) Tween-80/$CaCl_2$, pH7,0 (Ignjatovic and Dey, 1993); (iv) Tributyrin (glycerol tributyrate), pH7,0 (Fryer *et al.*, 1966). The plates were incubated for 5-10 days at 30°C. The growth medium for olive oil/Rhodamine B plates contained (per liter): 3g malt extract, 5g peptone, 3g yeast extract, 16g agar and 10ml olive oil [1% ($^v/_v$) final concentration]in distilled $H_2O$. The mixture was stirred and emulsified with a Branson Sonifier Cell Disrupter B-30 and the pH adjusted to 7,0. The medium was autoclaved and cooled to about 55°C and 10ml of filter-sterilised Rhodamine B solution [0,1% ($^w/_v$) final concentration] was added. The medium (20ml) was poured into sterile petri dishes.

Tween (20 and 80)/ $CaCl_2$ contained (per liter) : 3g malt extract, 5g peptone , 3g yeast extract, 16g agar, 10ml Tween 20 or Tween 80 [0,1% ($^v/_v$) final concentration] and 9mg $CaCl_2$ [0,8mM final concentration] in distilled $H_2O$. After the medium was adjusted to pH7,0, autoclaved and cooled to about 55°C, 20ml was poured into each sterile plastic petri dish.

The growth medium for tributyrin plates contained (per liter) : 3g malt extract, 5g peptone, 3g yeast extract, 16g agar and 10ml tributyrin [1% ($^v/_v$) final concentration] in distilled $H_2O$. The mixture was stirred and emulsified with a Branson Sonifier Cell Disrupter B-30 and the pH adjusted to 7,0. The medium was autoclaved and cooled to about 55°C. The medium (20ml) was poured into a sterile plastic petri dish.

### *3.2.1.2 Lipase production in liquid culture media*

Two lipase-producing black yeast isolates were chosen for further screening in liquid cultures containing different carbon sources as inducers of lipase production. Media containing (i) Tributyrin, (ii) Olive oil, (iii) Tween-80, (iv) Glucose was prepared as indicated in Table 3.1.

A standard LB-medium recipe was used, containing : 10g Triptone, 5g Yeast extract and 5g NaCl together with the inducers, in 900ml distilled $H_2O$.

Table 3.1 : **Inducers used in growth media.**

| | **Tributyrin (ml)** | **Olive oil (ml)** | **Tween-80 (ml)** | **Glucose (g)** | **Gum Arabic (g)** |
|---|---|---|---|---|---|
| (i) | 3,5 | - | - | - | 1,4 |
| (ii) | - | 3,5 | - | - | 1,4 |
| (iii) | - | - | 3,5 | - | - |
| (iv) | - | - | - | 3,5 | - |

The media containing tributyrin and olive oil were emulsified using a Branson Sonifier Cell Disrupter B-30.

### *3.2.1.3 Optimisation of lipase production*

Different methods were used for screening in liquid cultures to determine the optimum lipase production : (i) Inoculating directly from the plates and inducing after the cells in the conical flask were grown to their maximum. Aliquots were withdrawn every 30 minutes and centrifuged for 10 minutes in an Eppendorf-tube. The supernatants were assayed for lipase activity with the pNPP assay. (ii) The second method was to compare the lipase production of a young culture with that of an older culture that already produced lipase on the plates. Again, aliquots were taken every 30 minutes, centrifuged and then assayed with the pNPP assay. (iii) The enzyme production was determined using two different media recipes, namely (a) $H_2O$ and Tween-80/ olive oil and (b) standard YM-media and Tween-80/ olive oil. Aliquots were withdrawn, centrifuged and assayed with the pNPP assay. (iv) The lipase production was determined by inoculating using only a loop and then compared to the lipase production when inoculating using a whole plate ("lawn"-method). Aliquots were taken every 30 minutes, centrifuged for 10 minutes. The supernatant was assayed using the pNPP assay.

### 3.2.2 Enzyme assays and protein determination

#### *3.2.2.1 pNPP assay*

The assay method is based on the method used by Winkler and Stuckmann (1979) and determines both lipases and esterase activity. An artificial substrate was used to assay lipase activity, namely a *p*-nitrophenyl ester, *p*-nitrophenylpalmitate (*p*NPP, Figure 3.1). This assay measures the release of *p*-nitrophenol (*p*NP) from *p*NPP. Two solutions were prepared. Solution 1 contained *p*NPP (93mg) dissolved in propan-2-ol (30ml). Solution 2 contained sodium deoxycholate (984,2mg) and gum arabic (475mg) dissolved in 418ml buffer (50mM phosphate buffer, pH 8,5). The assay mixture was prepared by the dropwise addition of 1ml of solution 1 and 2,54ml propan-2-ol to 30ml of solution 2 with continuous stirring to obtain a stable emulsion. 25μl of the enzyme solution was then added to 600μl of the assay mixture in a 0,5ml plastic cuvette. The liberated *p*NP was photometrically measured at a wavelength of 410nm and a temperature of 37°C using a Beckman DU 650 spectrophotometer fitted with an Auto 6-Sampler (water-regulated) connected to a Haake D1-L heating bath/circulator (Fisons). The activity was calculated using kinetic software supplied by Beckman.

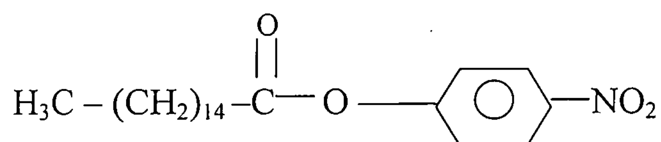



**Figure 3.1 : The structure of *p*-nitrophenyl palmitate**

Lipase activity in U/ml (umole FA's released.min$^{-1}$.ml$^{-1}$ enzyme) was calculated as follows:

$$U.ml^{-1} = \frac{V}{v \times \varepsilon \times d \times \Delta A.min^{-1}}$$

$$= \frac{0{,}60}{0{,}025 \times 15 \times 1 \times \Delta A.min^{-1}}$$

$$= 1{,}67 \times \Delta A.min^{-1}$$

| where V | : | Substrate volume (ml) |
|---|---|---|
| v | : | Enzyme volume (ml) |
| ε | : | Extinction coefficient of *p*NP at 410nm = 15 (L x $mmole^{-1}$ x $cm^{-1}$ = ml x $umole^{-1}$ x $cm^{-1}$) (Vorderwülbecke *et al.*, 1992) |
| d | : | Light path of cuvette (cm) |
| $\Delta A.min^{-1}$ | : | Change of absorbance per minute at 410nm |

One unit (U) of lipase activity is defined as the amount of enzyme that releases 1 μmole of fatty acid per minute.

*3.2.2.2 Copper olive oil assay*

"True" lipase activity was assayed as follows: a chloroform solution containing extracted fatty acids was shaken with a copper nitrite-triethanolamine reagent (Duncombe, 1963). Diethyldithiocarbamate, a reagent for the micro-determination of copper, was then added to the chloroform solution. Copper complexed with the fatty acids resulting in the formation of a yellow coloured complex which can be measured at 440nm.

The copper reagent contained $Cu(II)(NO_3)_2.3H_2O$ (16,125g) from Merck and triethylanolamine (32,5ml) from Merck (>99%) which was dissolved separately in distilled water (200ml). The solutions were combined, the pH adjusted to 7,5 and the volume adjusted to 500ml in a volumetric flask. The diethyldithiocarbamate reagent contained sodium diethyldithiocarbamic acid (0,25% $^w/_v$) in 2-butanol. A fresh solution had to be prepared daily. The olive oil emulsion, which acts as a substrate for the enzyme, consisted of olive oil (10% $^v/_v$) and gum arabic (1% $^w/_v$) dissolved in distilled water and emulsified with a Branson Sonifier Cell Disruptor B-30 until a milky emulsion was obtained. The assay buffer consisted of a 5mM TRIS-HCl buffer and $CaCl_2.2H_2O$ (0.04M) from Merck, pH8,5.

The lipase assay protocol was as follows: olive oil emulsion (0,5ml) and enzyme (0,1ml) were added to the assay buffer (0,5ml), mixed thoroughly and incubated for 60 minutes at 37°C in a shaking water bath. The reaction was stopped by the addition of copper reagent (2,5ml) and chloroform (5ml) to extract the fatty acids, mixed again for 20 minutes at 37°C in the shaking water

bath and centrifuged for 5 minutes at 1 000rpm. The upper, aqueous phase was removed by aspiration. A portion (2ml) of the chloroform solution was transferred to a clean tube and diethyldithiocarbamate reagent (0,5ml) was added. The absorbance of this solution was measured at 440nm against a blank which was exposed to the same procedure. The concentration of fatty acid (FA) in µmole.tube$^{-1}$ was read off the standard curve.

Calculation of lipase activity in U/ml (µmole FA's.min$^{-1}$.ml$^{-1}$ enzyme):

$$U.ml^{-1} = \frac{[FA]\mu mole.tube^{-1}}{60\ min \times 0{,}1ml.tube^{-1}}$$

$$= \frac{[FA]\mu mole.min^{-1}.ml^{-1}}{6}$$

$$= \mu mole\ FA's.min^{-1}.ml^{-1}$$

One unit (U) of lipase activity is defined as the amount of enzyme that releases 1µmole of fatty acid per minute.

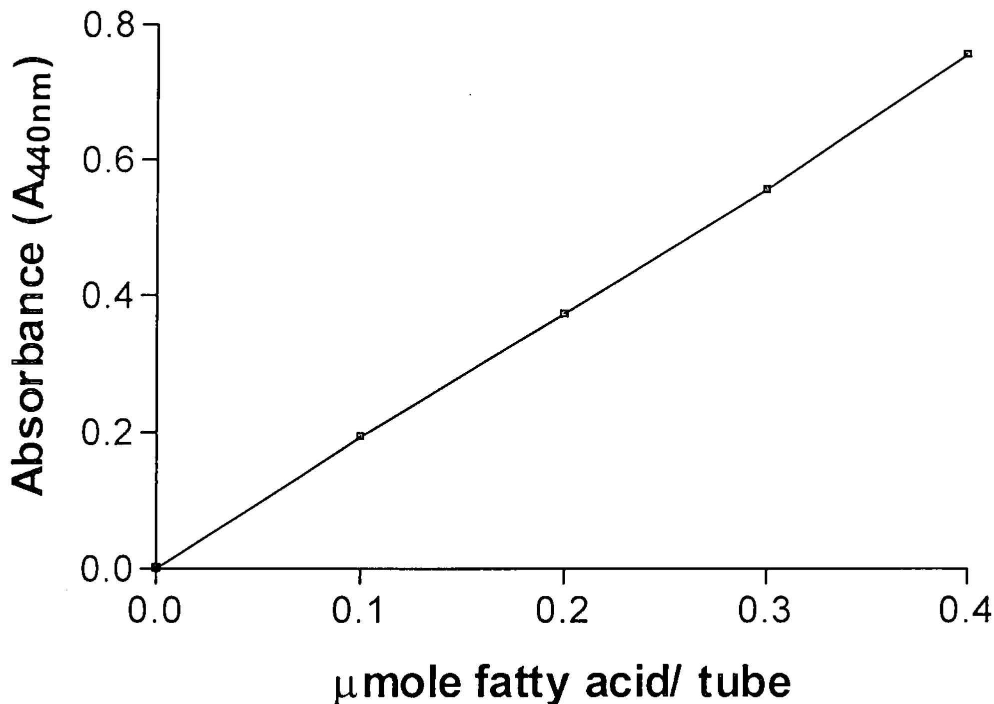



Figure 3.2: **Standard curve for assay of fatty acids released with the olive oil assay using stearic acid as standard. Standard deviations for the triplicate readings are shown.**

*3.2.2.3 pH-stat method*

The interfacial activation studies were done using the pH-stat method. This system was supplied by Metrohm and consists of an Impulsomat, a Metrohm Dosimat, a Metrohm pH meter and a 486 personal computer. An artificial substrate was used, namely tripropionin (ACROS ORGANICS, USA). The substrate and a TRIS-NaCl buffer (0,1mM NaCl, 1mM TRIS, pH9,0) was stirred well in a temperature-regulated cell. The assay was started by the addition of 200µl enzyme. When the enzyme cleaves the substrate molecule, a free fatty acid and alcohol is released. The acid lowers the pH and NaOH is added to keep the pH constant at 8,5.

***3.2.2.4 Protein determination***

Protein concentrations were estimated either by absorbance at 280nm or by using the bicinchoninic acid (BCA) method (Smith *et al.*, 1985) which was supplied as a kit by Pierce, Rockford, IL, USA. Both the standard and micro assay kits were used.

The BCA protein assay reagent is a highly sensitive reagent for the spectrophotometric determination of protein concentration. The reagent system combines the biuret reaction of protein with $Cu^{2+}$ in an alkaline medium which yields $Cu^{1+}$, with BCA, which is a sensitive, stable and highly selective detection reagent for $Cu^{1+}$. A purple reagent product is formed by the interaction of two molecules of BCA with one $Cu^{1+}$ ion. The reaction product is water soluble and exhibits strong absorbance at 562nm (Figure 3.2). According to Wiechelman *et al.* (1988) the macromolecular structure of protein and the four amino acids, cysteine, cystine, tryptophan and tyrosine, have been reported to be responsible for colour formation in protein samples when assayed with BCA.

The methods used were those supplied with the commercially available kits. A set of protein standards was prepared with bovine serum albumin (BSA), provided by Pierce as part of the BCA protein assay kit, in the range of 20-120µg/ml for the standard protein assay and 1-10µg/ml for the micro protein assay. Standard curves were prepared and used to determine the protein concentration of unknown protein samples (Figures 3.3 and 3.4).

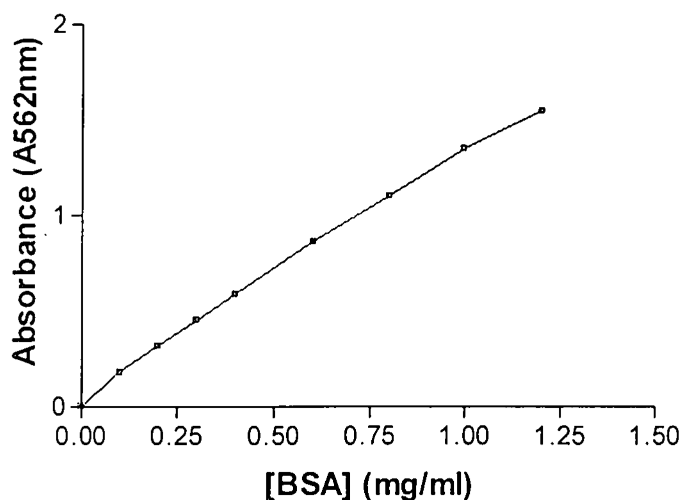



Figure 3.3: **Standard curve for BCA protein assay with BSA as protein standard. Standard deviations of triplicate readings are shown.**

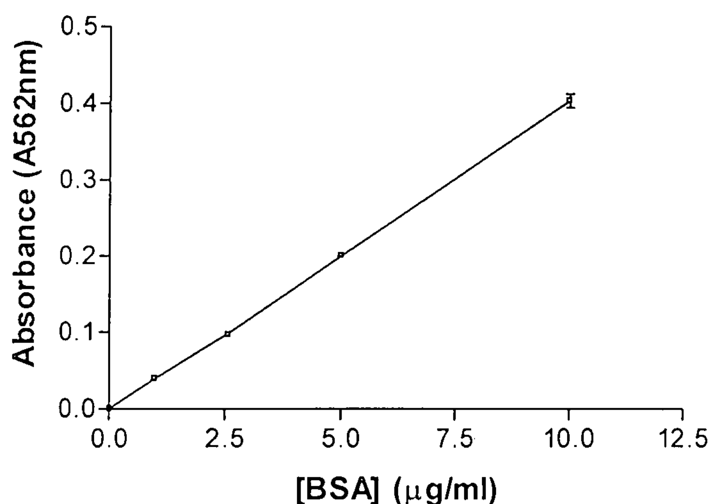



Figure 3.4 : **Standard curve for the Micro BCA protein assay with BSA as protein standard. Standard deviations of triplicate readings are shown.**

## 3.2.3 Electrophoresis

### *3.2.3.1 SDS-PAGE*

Sodium dodecyl sulphate polyacrylamide gel electophoresis (SDS-PAGE) on 12% resolving gels and 4% stacking gels was used to monitor the purification process, to estimate the homogeneity of purified fractions and to determine relative molecular mass ($M_r$) of the lipase by comparing its electrophoretic mobility with those of standard proteins with known molecular masses.

Protein samples which were too diluted for direct loading on SDS-PAGE were concentrated using drying under vacuum in a Savant Speed Vac Condensation Trap or air dried in a dialysis bag.

SDS-PAGE was performed using the "Mighty Small" miniature slab gel electrophoresis unit, SE 200 from Hoefer Scientific Instruments. Electrophoresis was performed on approximately 10μg protein (20μl of the prepared sample). The protocol used was a slight modification of the procedure of Laemmli (1970). The protein bands were detected with either Coomassie Brilliant Blue R-250, silver staining or both. Staining with Coomassie Blue was carried out as described in the Hoefer instruction manual and silver staining was performed according to the method described by Switzer *et al.*, (1979).

The protein standards used were α2-Macroglobulin ($M_r$ 170 000), β-galatosidase ($M_r$ 116 400), Fructose-6-phosphate kinase ($M_r$ 85 200), glutamate dehydrogenase ($M_r$ 55 600), aldolase ($M_r$ 39 200), triose phosphate isomerase ($M_r$ 26 600), trypsin inhibitor ($M_r$ 20 100) and lysozyme ($M_r$ 14 300), (relative molecular mass, $M_r$, in parenthesis).

### 3.2.4 Development of purification protocol

Because of the lack of articles describing the purification of yeast lipases, in particular lipases produced by black yeasts, it was decided to develop a purification protocol specific for the *Exophiala dermatiditis* lipases. A number of different isolation procedures were performed and assessed. Unless mentioned otherwise, lipase activity was determined with the standard pNPP assay method as described in section 3.2.2.1.

#### *3.2.4.1 Purification of Exophiala dermatiditis UOFS Y-2044 lipase (ED2044L)*

##### 3.2.4.1.1 Assessment of binding to MIMETIC A6XL dye adsorbent ligands using the Piksi kit

Growth medium (1L) containing olive oil as carbon source was prepared as in section 3.2.1.2. Olive oil was used as carbon source due to the fact that it was the best lipase inducer (Results section 4.1.3). The cultivation was started by inoculating using a whole plate as this method resulted in the highest lipase production. Cultivation was continued for 2-3h and monitored until the lipase production peaked. The culture was centrifuged at 22 000xg at 4°C for 60 minutes to remove the cell mass. The supernatant, containing the crude extracellular lipase, was decanted. PMSF (0.0871g in 5ml ethanol), was added to the supernatant (final concentration 0,1M) to inhibit protease activity. The supernatant was used in further isolation steps.

Since binding to the MIMETIC A6XL adsorbent ligands is influenced by buffer conditions it was decided to use SEC on HW50F (100cm x 2,5cm) as a buffer change step. Enzyme samples in the different buffers, namely 0,1M TRIS-HCl (pH8,5); 0,025M phosphate buffer (pH6,0) and 0,01M phosphate buffer (pH5,8) were then used to assess binding on the Piksi kit (a kit consisting of 10 x 1ml columns of each of the 10 MIMETIC A6XL dye adsorbent ligands and manufactured by Affinity Chromatography Ltd., and supplied locally by SEPARATIONS).

Maximal binding and yields were obtained with a 0,01M phosphate buffer (pH5,8) on the MIMETIC Red A6XL and Yellow A6XL dye adsorbent ligands. A further assessment was then carried out on packed columns of MIMETIC Red A6XL and MIMETIC Yellow A6XL dye adsorbent ligands.

### 3.2.4.1.2 First isolation attempt

Cultivation of the organism was performed as in section 3.2.4.1.1. Centrifugation was performed under the same conditions as in section 3.2.4.1.1. PMSF was added to the supernatant.

The enzyme fraction was applied to a pre-equilibrated (0,01M phosphate buffer, pH5,8) Toyopearl HW50F column (100cm x 2,5cm) at a flow rate of $15ml.h^{-1}$. The enzyme was eluted with the same phosphate buffer. Fractions (5ml) were collected. The fractions were monitored for absorbance at 280nm ($A_{280nm}$), assayed for lipase activity and the active fractions were pooled and used to assess binding on the MIMETIC Yellow A6XL dye adsorbent ligand affinity column.

The active enzyme fraction was applied to the pre-equilibrated (0,01M phosphate buffer, pH5,8) MIMETIC Yellow A6XL dye adsorbent ligand affinity column (10cm x 2,5cm) at a flow rate of $15ml.h^{-1}$. After application of the sample to the column, it was washed with the same buffer until the $A_{280nm}$ readings were $<0.02$. Elution was performed by using 200ml of 0-2M KCl gradient in 0,01M Phosphate buffer (pH5,8). $A_{280nm}$ and lipase activity were determined for the 5ml fractions collected. The active fractions were pooled and protein concentration determined. The MIMETIC Yellow A6XL dye adsorbent ligand was regenerated using a 0,01M phosphate buffer (pH5,8) containing 1M NaOH followed by equilibration with a 0,01M phosphate buffer (pH5,8).

### 3.2.4.1.3 Second isolation attempt

Cultivation of the organism was performed as in section 3.2.4.1.1. Centrifugation was performed under the same conditions as in section 3.2.4.1.1. PMSF was again added to the supernatant.

The enzyme fraction was applied to the pre-equilibrated (0,01M phosphate buffer, pH5,8) Toyopearl HW50F size exclusion column (100cm x 2,5cm) at a flow rate of $15ml.h^{-1}$. The enzyme was eluted with the same phosphate buffer. Fractions (5ml) were collected. The fractions were monitored for absorbance at 280nm, assayed for lipase activity and the active fractions were pooled and used to assess binding on the MIMETIC Red A6XL dye adsorbent ligand affinity column.

The active enzyme fraction was applied to the MIMETIC Red A6XL dye adsorbent ligand affinity column (10cm x 2,5cm) at a flow rate of $15ml.h^{-1}$. After application of the sample to the column, it was washed with the same buffer until the $A_{280nm}$ readings were <0.02. Elution was performed by using 200ml of 0-2M KCl gradient in 0,01M phosphate buffer (pH 5,8). Fractions (5ml) were collected. $A_{280nm}$ and lipase activity were determined for the fractions collected. The active fractions were pooled and protein concentration determined. The MIMETIC Red A6XL dye adsorbent ligand was regenerated using a 0,01M phosphate buffer (pH5,8) containing 1M NaOH followed by equilibration with a 0,01M phosphate buffer (pH5,8).

### 3.2.4.1.4 Third isolation attempt

The cultivation, centrifugation and addition of PMSF were performed as in section 3.2.4.1.1. 200ml of the crude enzyme was used in an ethanol precipitation step. Two volumes of ice-cold ethanol were added to 200ml of supernatant over a period ten minutes. The mixture was stirred for one hour on ice. After this the mixture was centrifuged at 22 000xg for 30 minutes at 4°C. The supernatant was discarded and the pellet was dissolved in 40ml of buffer. This was again centrifuged at 22 000xg for 30 minutes at 4°C. The supernatant was assayed for lipase activity.

The active fraction was applied to a pre-equilibrated (0,01M TRIS-HCl, pH8,0) Toyopearl DEAE-650M column (12cm x 2,5cm) at a flow rate of $60ml.h^{-1}$, washed with 0,01M TRIS-HCl buffer (pH8,0) until the $A_{280nm}$ reached <0,02 and eluted with 200ml gradient (0-2M KCl) of 0,01M TRIS-HCl (pH8,0). Fractions (5ml) were collected, monitored for absorbance at 280nm, assayed

for lipase activity and active fractions pooled. The sample was stored at 4°C for protein determination and SDS-PAGE.

#### 3.2.4.1.5 Fourth isolation attempt

Cultivation of the organism was performed as in section 3.2.4.1.1. Centrifugation of the crude fraction was done as described in section 3.2.4.1.1 and the protease inhibitor, PMSF, was added under the conditions described in section 3.2.4.1.1.

Crude enzyme (40ml) was applied to a pre-equilibrated (50mM phosphate buffer, pH5,8) Toyopearl SP-650M column (25cm x 2,5cm) at a flow rate of $60ml.h^{-1}$, washed with 50mM phosphate buffer (pH5,8) until the $A_{280nm}$ reached <0,02, and eluted with 200ml gradient (0-2M KCl) of 0,01M TRIS-HCl (pH8,0). Fractions (5ml) were collected and monitored for absorbance at 280nm, assayed for lipase activity and active fractions were pooled. The sample was stored at 4°C for protein determination and SDS-PAGE. If necessary, all ion-exchange media were regenerated using 0,5M NaOH before equilibration.

#### 3.2.4.1.6 Fifth isolation attempt

The cultivation of organism, centrifugation of crude fraction and addition of PMSF were performed as in section 3.2.4.1.1.

Crude enzyme (40ml) was applied to a pre-equilibrated (50mM phosphate buffer, pH8,0) Toyopearl Super-Q 650S column (25cm x 2,5cm) at a flow rate of $60ml.h^{-1}$, washed with 50mM phosphate buffer (pH8,0) until the $A_{280nm}$ reached <0,02 and eluted with 200ml of a 0-2M KCl gradient in 50mM phosphate buffer (pH8,0). Fractions (5ml) were collected, monitored for absorbance at $A_{280nm}$ and assayed for lipase activity. The active fractions were pooled.

#### 3.2.4.1.7 Sixth isolation attempt

Cultivation and centrifugation were done as in section 3.2.4.1.1. The protease inhibitor was added under the same conditions as in section 3.2.4.1.1.

The crude enzyme (40ml) was applied onto the pre-equilibrated Toyopearl SP-650M column and eluted as previously described in section 3.2.4.1.5. The active non-binding fractions were pooled

and applied onto a pre-equilibrated Toyopearl Super-Q 650S column. The column was washed with 50mM phosphate buffer (pH8,0) until the $A_{280nm}$ reached a value <0,02 and enzyme activity was eluted with a 300ml 0-2M KCl gradient in 50mM phosphate buffer (pH8,0). Fractions (5ml) were collected. The fractions were monitored for absorbance at $A_{280nm}$, assayed for lipase activity and active fractions were pooled.

### 3.2.4.1.8 Seventh isolation attempt

Cultivation of the organism was done as described in section 3.2.4.1.1. The centrifugation of the crude fraction was done as in section 3.2.4.1.1. PMSF was added as protease inhibitor under the same conditions as described in section 3.2.4.1.1.

Crude enzyme (100ml) was applied onto the previously equilibrated Toyopearl SP-650M column. After application of the fraction, the column was washed with a 50mM phosphate buffer (pH5,8) until the $A_{280nm}$ values were <0,02. Fractions (5ml) collected, were monitored for absorbance at 280nm and assayed for lipase activity. The active fractions were pooled.

The active fraction from the Toyopearl SP-650M column was applied onto the pre-equilibrated Toyopearl Super-Q 650S column. After application of the sample to the column, it was washed with a 50mM phosphate buffer (pH 8,0) until the $A_{280nm}$ readings were <0,02. Elution was performed by using 200ml of a 0-2M KCl gradient in 0,05mM phosphate buffer (pH 8,0). Fractions (5ml) were collected. $A_{280nm}$ and lipase activity were determined for the fractions collected. The active fractions were pooled.

The active fraction obtained from the Toyopearl Super-Q 650S column was applied onto the pre-equilibrated Toyopearl HW50F column and eluted with 0,01mM phosphate buffer, (pH5,8). The enzyme was eluted with the same buffer. Fractions (5ml) were collected, monitored for absorbance at 280nm, assayed for lipase activity and active fractions pooled.

The pooled enzyme fraction from the Toyopearl HW50F step was then applied to the pre-equilibrated (0,01M phosphate buffer, pH5,8) MIMETIC Red A6XL dye adsorbent ligand affinity column (10cm x 2,5cm) at a flow rate of $15ml.h^{-1}$. The column was then washed with the same buffer until the $A_{280nm}$ readings were <0,02. Elution was then performed by using a 200ml gradient of 0-2M KCl gradient in 0,01M Phosphate buffer (pH 5,8). Fractions (5ml) were collected. $A_{280nm}$

and lipase activity were determined for the fractions collected. The active fractions were pooled and used for protein determination , SDS-PAGE and further characterisation.

### *3.2.4.2 Purification of Exophiala dermatiditis UOFS Y-2048 lipase (ED2048L)*

#### 3.2.4.2.1 Assessment of binding to MIMETIC A6XL dye adsorbent ligands

Crude enzyme was obtained as described in section 3.2.4.1.1.

Crude enzyme (200ml) was applied to the pre-equilibrated (0,01M phosphate buffer, pH5,8) Toyopearl HW50F column (100cm x 2,5cm) at a flow rate of $15ml.h^{-1}$. Fractions (5ml) were collected, monitored for absorbance at 280nm and assayed for lipase activity. The pooled active fraction was then applied to the pre-equilibrated (0,01M phosphate buffer, pH5,8) MIMETIC Yellow A6XL dye adsorbent ligand affinity column (10cm x 2,5cm) at a flow rate of $15ml.h^{-1}$. The column was washed until the $A_{280nm}$ readings were <0,02. Elution was performed by using a 200ml gradient of 0-2M KCl in 0,01M phosphate buffer (pH 5,8). $A_{280nm}$ and lipase activity were determined for each of the 5ml fractions collected. The active fractions were pooled and protein concentration determined.

The afore-mentioned was then repeated to assess binding on the MIMETIC Red A6XL dye adsorbent ligand affinity column.

#### 3.2.4.2.2 Purification protocol

Cultivation of the organism was performed as in section 3.2.4.1.1. Centrifugation was performed under the same conditions as in section 3.2.4.1.1. PMSF was again added to the supernatant.

ED2048L was purified using the same protocol developed for ED2044L except for the final affinity chromatography step where MIMETIC Yellow A6XL dye adsorbent ligand was substituted for MIMETIC Red A6XL dye adsorbent ligand.

The active enzyme fraction was applied onto the previously equilibrated Toyopearl SP-650M column. After application of the fraction, the column was washed with a 50mM phosphate buffer (pH5,8) until the $A_{280nm}$ values were <0,02. The fractions (5ml) collected, were monitored for absorbance at 280nm and assayed for lipase activity. The active fractions were pooled.

The active fraction from the Toyopearl SP-650M column was applied onto the Toyopearl Super-Q 650S column, which was regenerated and equilibrated as described in section 3.4.2.1.7. After application of the sample to the column, it was washed with a 50mM phosphate buffer (pH 8,0) until the $A_{280nm}$ readings were <0,02. Elution was performed by using 200ml of a 0-2M KCl gradient in 50mM phosphate buffer (pH 8,0). Fractions (5ml) were collected. $A_{280nm}$ and lipase activity were determined for the fractions collected. The active fractions were pooled.

The active enzyme fraction was applied to the pre-equilibrated (0,01M phosphate buffer, pH5,8) Toyopearl HW50F size exclusion column (100cm x 2,5cm) at a flow rate of 15ml.h-1. The enzyme was eluted with the same phosphate buffer. Fractions (5ml) were collected. The fractions were monitored for absorbance at 280nm, assayed for lipase activity and the active fractions were pooled and used to assess binding on the MIMETIC Yellow A6XL dye adsorbent ligand affinity column.

The active enzyme fraction was applied to the MIMETIC Yellow A6XL dye adsorbent ligand Affinity column (25cm x 2,5cm) at a flow rate of 15ml.$h^{-1}$. The column was regenerated using a 0,01M phosphate buffer (pH 5,8) containing 1M NaOH. The column was equilibrated with a 0,01M phosphate buffer (pH 5,8). After application of the sample to the column, it was washed with the same buffer until the $A_{280nm}$ readings were <0,02. Elution was performed by using 200ml of a 0-2M KCl gradient in 0,01M Phosphate buffer (pH 5,8). $A_{280nm}$ and lipase activity were determined for the 5ml fractions collected.

Active fractions from the final purification step were pooled and used for a limited characterisation of the enzyme with the aim of confirming whether ED2044L and ED2048L were the same enzyme.

### 3.2.5 Physical-chemical characterisation

All characterisation studies were done on the final enzyme fraction resulting from the purification procedures described in sections 3.2.4.1.8 and 3.2.4.2.2. Enzyme activity was measured using the *p*NPP assay (section 3.2.2.1) at 37°C in a Beckman DU 650 spectrophotometer fitted with an Auto 6-Sampler (water-regulated) connected to a Haake D1-L heating bath/ circulator (Fisons). Temperature was varied in the case of the optimum temperature experiment.

### *3.2.5.1 Thermostability*

Thermostability of the purified lipase was determined at the following temperatures; 20°C, 30°C, 40°C, 50°C, 60°C, 70°C and 80°C. The enzyme was incubated at the different temperatures for different time intervals and aliquots were withdrawn and immediately placed in ice-cold water and then on ice. Assays were done in triplicate and the experiment was repeated three times on different days. The first activity reading at t=0, was taken as 100% activity.

### *3.2.5.2 Optimum temperature*

The optimum temperature was determined over a range of 20°C-80°C. In each case, the assay mixture was equilibrated at the required temperature before the addition of enzyme. Assays were done in triplicate together with a blank rate at each temperature, which was subtracted from each value. The experiment was repeated three times on three different days. Standard deviations were calculated for each temperature to indicate the daily variation.

### *3.2.5.3 Optimum pH*

The optimum pH was determined over a range of pH 5-11. The pH range was constructed by adjusting the pH of the assay buffers used. Between pH 5 and pH 6,5, a 0,1M TRIS-Glycine buffer was used. Between pH 6.5 and pH 9, a 0,05M phosphate buffer was used and between pH 9 and pH 11, a 0,5M Glycine-NaOH buffer was used. The rest of the assay procedure was the same as in section 3.2.2.1. Enzyme assays were done in triplicate together with a control at each pH. The experiment was repeated three times on three different days.

### *3.2.5.4 pH stability*

The pH of the enzyme solution was adjusted to the required value, using a strong acid or base. The pH stability was determined over a range of pH 5-12. The enzyme solution was incubated at 30°C for 60 min and the remaining activity assayed in triplicate. The experiment was repeated three times on three different days.

### *3.2.5.5 Substrate specificity*

The fatty acid preference of the lipase was determined using *p*-nitrophenyl esters of the following fatty acids: propionic acid (3:0), butyric acid (4:0), caproic acid (6:0), caprylic acid (8:0), capric acid (10:0), lauric acid (12:0), myristic acid (14:0), palmitic acid (16:0), palmitoleic acid (16:1), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2) and α-linolenic acid (18:3). The substrate concentrations were adjusted to the same value by measuring A280nm. The activity was assayed as previously described with *p*NPP being substituted by the above-mentioned *p*-nitrophenyl esters.

Substrate specificity was also determined using commercial triacylglycerols (TAG's). The following TAG's were used: triacetin (2:0), tripropionin (3:0), tributyrin (4:0), triolein (18:1), trilinolein (18:2 (cis)) and trieliadin (18:2 (trans)). The activity was assayed using a Metrohm pH-stat system (Vorderwülbecke *et al.*, 1992), which consisted of an impulsomat, a dosimat, a pH meter and a personal computer and fitted with a temperature-regulated stirred cell. Two solutions were prepared. Solution 1 contained 3ml of the TAG added to 15ml Gum Arabic solution (20,6g Gum Arabic dissolved in 200ml $H_20$). Solution 2 was a 0,1mM NaCl and 1mM TRIS buffer. The assay mixture was prepared by adding 18,75ml of the buffer to 3,75ml of solution 1. This was stirred in the pH-stat. Adding 200µl of enzyme started the reaction. The reaction was run for 5min at 37°C, and titrated at pH8,5 with standard 0,025M NaOH, whereafter the average slope was determined. The slope was converted to activity (U = mmole/min/ml). One unit (U) of lipase activity is defined as one mmole of fatty acids per minute per ml enzyme. The activity was assayed in triplicate on three different days. The standard deviations were calculated.

### *3.2.5.6 Substrate concentration dependence*

Interfacial activation studies were done using tripropionin (ACROS ORGANICS, USA) as substrate. A TRIS-NaCl buffer (0,1mM NaCl, 1mM TRIS, pH9,0) was used. The assays were done using the pH-stat system (section 3.2.5.5). Substrate concentrations of 4.65mM to 186mM were prepared as shown in Table 3.2.

Table 3.2: **Preparation of assay solution with varying concentrations of tripropionin.**

| Tripropionin (mM) | 4,65 | 9,3 | 18,6 | 46,5 | 93,0 | 139,5 | 186,0 |
|---|---|---|---|---|---|---|---|
| Buffer (ml) | 22,475 | 22,45 | 2240 | 22,25 | 22,00 | 21,75 | 21,50 |
| Tripropionin (ml) | 0,025 | 0,050 | 0,10 | 0,25 | 0,50 | 0,75 | 1,00 |

The assay was done at pH 8,5, which was the optimum pH of the two lipases. The reaction was run for 5 min at 37°C, and titrated with standard 0,025M NaOH, whereafter the slope was determined.

### *3.2.5.7 Effect of detergents*

The effect of detergents on lipase activity was tested at concentrations below and above the critical micelle concentration (cmc = the minimum concentration at which detergents begin to form micelles). The standard pNPP assay mixture contained 0,25% (w/v) of an anionic detergent, sodium deoxycholate which was replaced with 0,25% (w/v) of the following: (i) another anionic detergent, SDS, (ii) a cationic detergent, Cetrimide (Hexadecyltrimethylammonium bromide; Fluka), (iii) the non-ionic detergents, Triton-X-100, Tween-80 and (iv) the zwitterionic detergent, CHAPS. The activity was measured in triplicate on three different days.

### *3.2.5.8 Effect of metal ions*

The effect of metal ions on lipase activity was assessed using $Ca^{2+}$, $Mg^{2+}$, $Fe^{3+}$, $Sn^{2+}$, $Hg^{2+}$, $Ba^{2+}$, $Cu^{2+}$ and $Mn^{2+}$ in the form of metal chlorides. A metal ion concentration range of 0-1,0mM was tested. Metal solutions were made 10 times more concentrated in water than the final concentration in the Eppendorf-tube while the assay mixture was made up 10% more concentrated than usual. The assay solution was then diluted with metal in the ratio of 11:1. Enzyme assays were done as usual with a blank rate for each metal concentration. The experiment was done in triplicate on different days.

### *3.2.5.9 Effect of EDTA*

A concentration range of 0-5mM EDTA was used. The enzyme was incubated with different concentrations of EDTA in Eppendorf-tubes for 30min at 37°C and the remaining activity assayed in triplicate together with a blank rate (enzyme incubated without EDTA). The activity of the

i 148 799 55

lowest concentration (0,0mM) was taken as 100%. The experiment was done in triplicate on three different days.

### *3.2.5.10. Effect of PMSF*

A PMSF concentration range of 0-2mM was investigated. The enzyme was incubated with different concentrations of PMSF for 30min at 37°C and the remaining activity was assayed in triplicate together with a blank rate (enzyme incubated without PMSF). The experiment was repeated on three different days.

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 Screening for lipase production

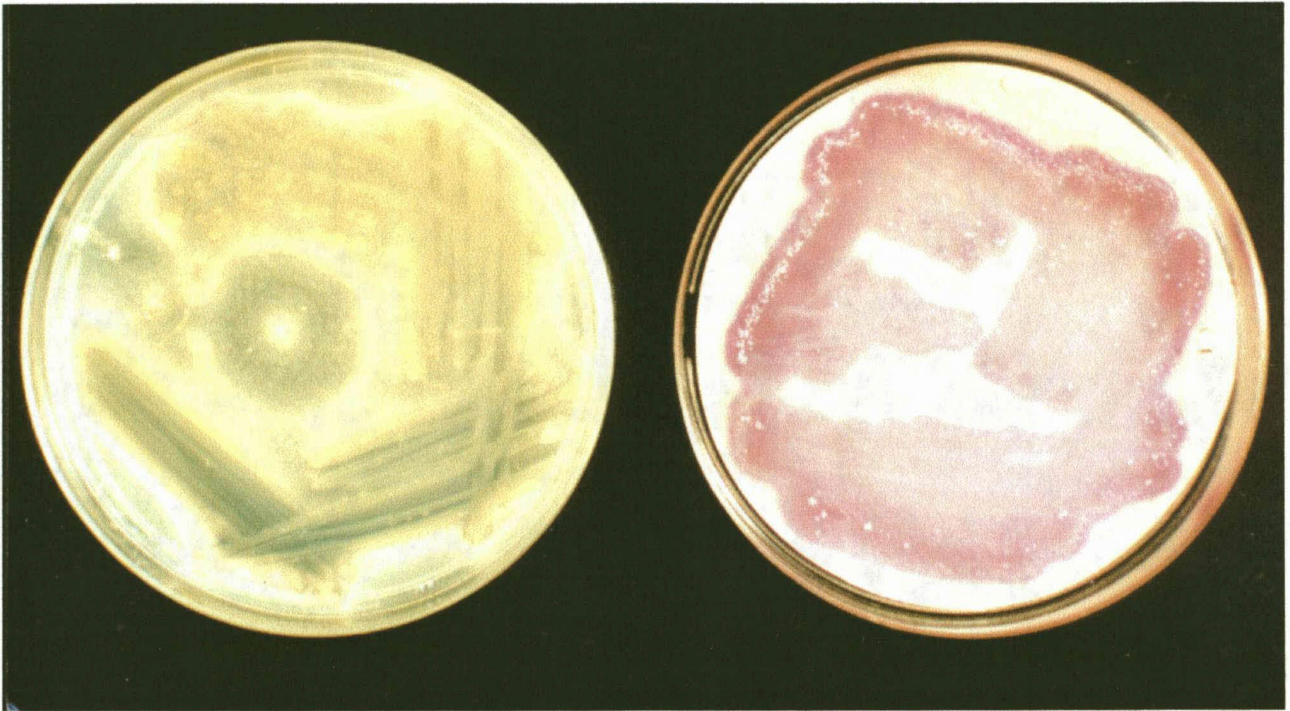
### 4.1.1 Lipase production on agar plates

Black yeast isolates (23), obtained from the culture collection of the Department of Microbiology and Biochemistry, were screened for lipase production on agar plates containing: (i) Rhodamine B/ olive oil, (ii) Tween-20/ $CaCl_2$, (iii) Tween-80/ $CaCl_2$ and (iv) tributyrin (Figure 4.1).

The Rhodamine B/ olive oil agar plates resulted in pink zones around the lipase positive colonies. Under UV light, orange fluorescent halos surrounded the colonies, an indication of extracellular lipase production. The Tween-20/ $CaCl_2$ or Tween-80/ $CaCl_2$ agar plates resulted in white opaque halos around the lipase positive colonies. The halos were the result of precipitation of $Ca^{2+}$ salts of fatty acids. Lipase-producing organisms on agar plates containing tributyrin were identified by zones of clearance around the colonies. The natural opacity of tributyrin agar plates is due to the presence of micro-droplets of tributyrin. Lipolytic organisms convert these into water-soluble butyric acid, so removing the opacity.

Of the 23 black yeast isolates screened, all 23 isolates showed lipase production on at least three different inducers and two were selected for further studies (Table 4.1).

(i) (ii)



(iii)

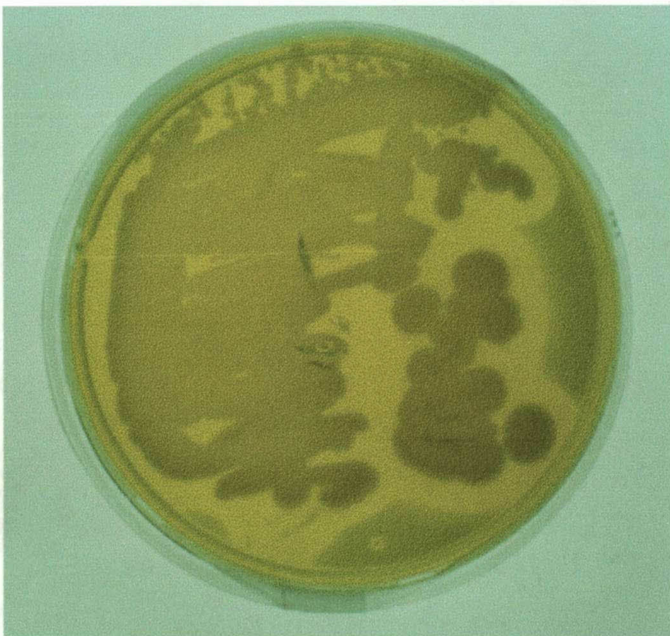


**Figure 4.1:** **Photographs showing a lipase-positive isolate growing on agar plates containing (i) Rhodamine B/ olive oil, (ii) Tween-80/ $CaCl_2$ and (iii) tributyrin.**

Table 4.1: Screening results of 23 black yeast isolates on agar plates containing: (i) Tween-80/ $CaCl_2$ or Tween-80/ $CaCl_2$, (ii) Rhodamine B/ olive oil and (iii) Tributyrin. (*) Indicates low lipase production, (**) indicates medium lipase production and (***) indicates very high lipase production.

| Media | Tween-80/ $CaCl_2$ | Tween-20/ $CaCl_2$ | Tributyrin | Rhodamine B/ olive oil |
|---|---|---|---|---|
| **Yeast** | | | | |
| T36 (VDW 91) | *** | *** | *** | *** |
| T29 (VDW 61) | *** | *** | *** | *** |
| **T20 (VDW 64)** | *** | *** | *** | *** |
| T64 (VDW 65) | ** | * | *** | ** |
| T95 (VDW 66) | ** | *** | *** | *** |
| T70 (VDW 67) | *** | *** | *** | *** |
| T17 (VDW 68) | *** | *** | *** | *** |
| T75 (VDW 69) | ** | * | ** | * |
| T16 (VDW 70) | No activity | No activity | * | No activity |
| T21 (VDW 71) | * | ** | * | * |
| T63 (VDW 72) | ** | * | No activity | * |
| T66 (VDW 73) | * | * | ** | * |
| T74 (VDW 74) | ** | No activity | ** | * |
| T69 (VDW 75) | ** | No activity | ** | * |
| T82 (VDW 76) | * | No activity | * | * |
| **T84 (VDW 77)** | * | * | ** | * |
| T88 (VDW 78) | ** | * | ** | * |
| T89 (VDW 79) | * | * | ** | * |
| T92 (VDW 80) | *** | *** | *** | *** |
| T97 (VDW 81) | ** | * | * | * |
| T98 (VDW 82) | * | * | ** | * |
| T103 (VDW 83) | * | ** | * | * |
| T102 (VDW 84) | *** | *** | *** | *** |
| T99 (VDW 85) | *** | *** | ** | ** |
| T11 | *** | *** | *** | *** |

Footnote: The two isolates highlighted in bold were selected for further study.

### 4.1.2 Lipase production in liquid culture media

Two of the black yeast isolates, T20 and T84, were selected for further studies and identified as *Exophiala dermatiditis* UOFS Y-2044 (ED2044L) and *Exophiala dermatiditis* UOFS Y-2048 (ED2048L), respectively. *Exophiala dermatiditis* UOFS Y-2044 (T20) was chosen because of its high lipase production on the different agar plates. *E. dermatiditis* UOFS Y-2048 (T84) was chosen because of its high phospholipase production (data not shown). It was initially decided to purify ED2044L and then to purify the phospholipase from *E. dermatiditis* UOFS Y-2048. However, *E. dermatiditis* UOFS Y-2048 took six weeks to produce phospholipase on an agar plate and, in contrast to the screening study done on agar plates, it produced high levels of lipase activity in liquid cultures. It was then decided to purify both the lipases. The phospholipase purification can be a possible future study.

The isolates were grown in shake cultures containing different carbon sources as inducers of lipase production, namely (i) Tributyrin, (ii) Olive oil, (iii) Tween-80 and (iv) Glucose.

Enzyme production was monitored over a period of 30h using the *p*NPP assay. Olive oil was the best inducer for both enzymes (Figure 4.2 (a), (b)) while glucose, Tween-80 and tributyrin resulted in almost no enzyme activity. From the enzyme activity profile with olive oil as carbon source, ED2044L was secreted immediately and reached a maximum after only three hours. ED2048L was also secreted immediately and reached a maximum after three and a half hours. The lipase activity produced by both enzymes was relative high although the activity of ED2048L was higher. Copper-Olive oil assays done on both the enzymes (data not shown) confirmed that ED2044L and ED2048L were both true lipases.

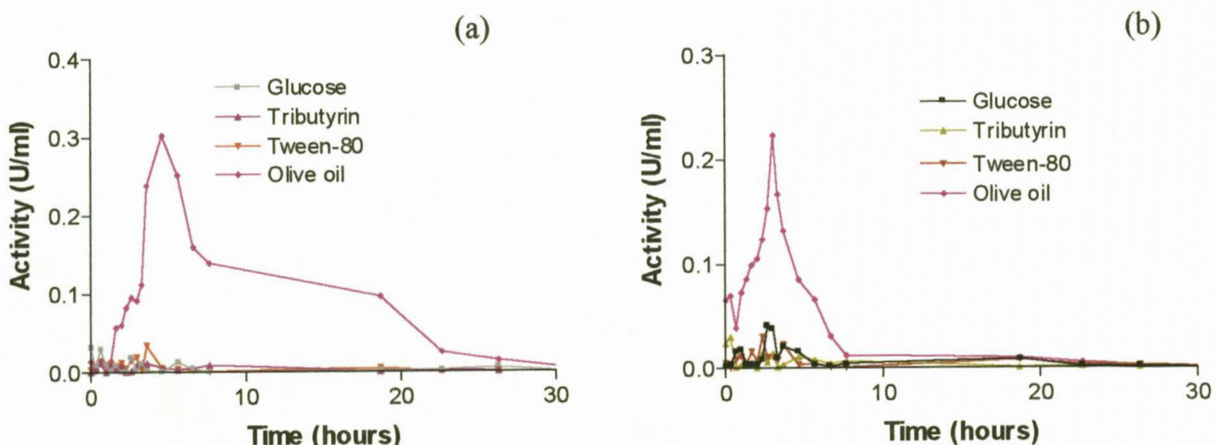



**Figure 4.2: Time-dependent lipase production on different carbon sources monitored with the *p*NPP assay. (a) Profile for ED2044L and (b) profile for ED2048L.**

### 4.1.3 Optimisation of lipase production

(i) The results of two methods of inoculating are illustrated in Figures 4.2 and 4.3. Figure 4.3 is the result of inducing the culture with olive oil whereas Figure 4.2 is obtained by inoculating directly from an agar plate. Figure 4.3 shows that the activity of both the lipases reached a maximum immediately after inoculating whereas in Figure 4.2 the maximum was reached after three hours with higher activity being observed. This led to the conclusion that it is not necessary to let the cells grow before lipase is produced.

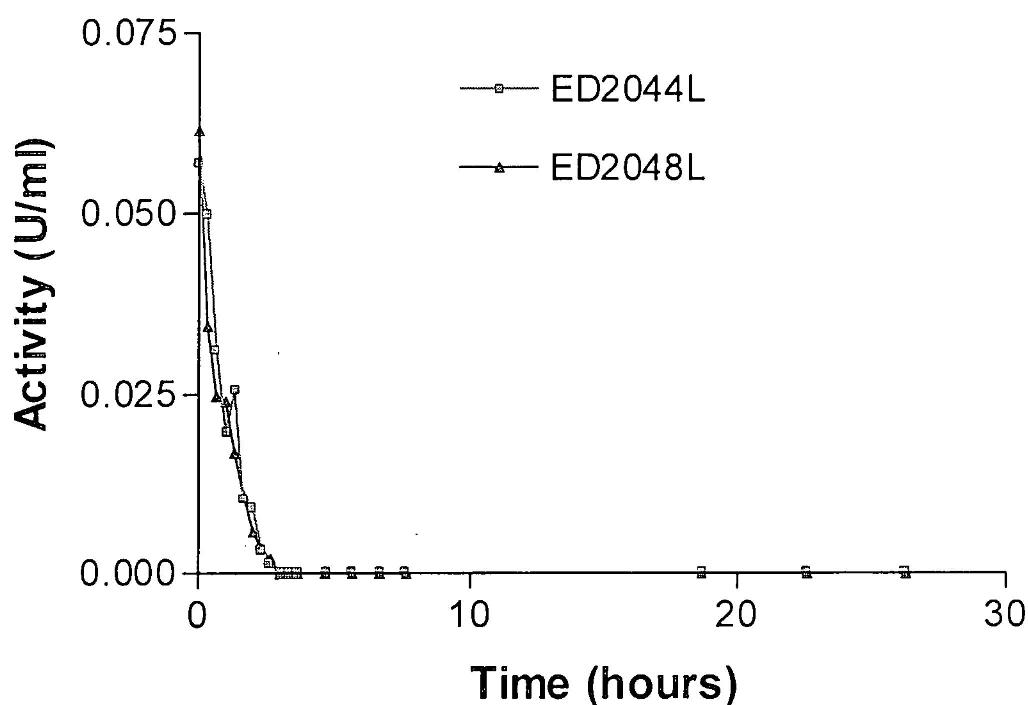
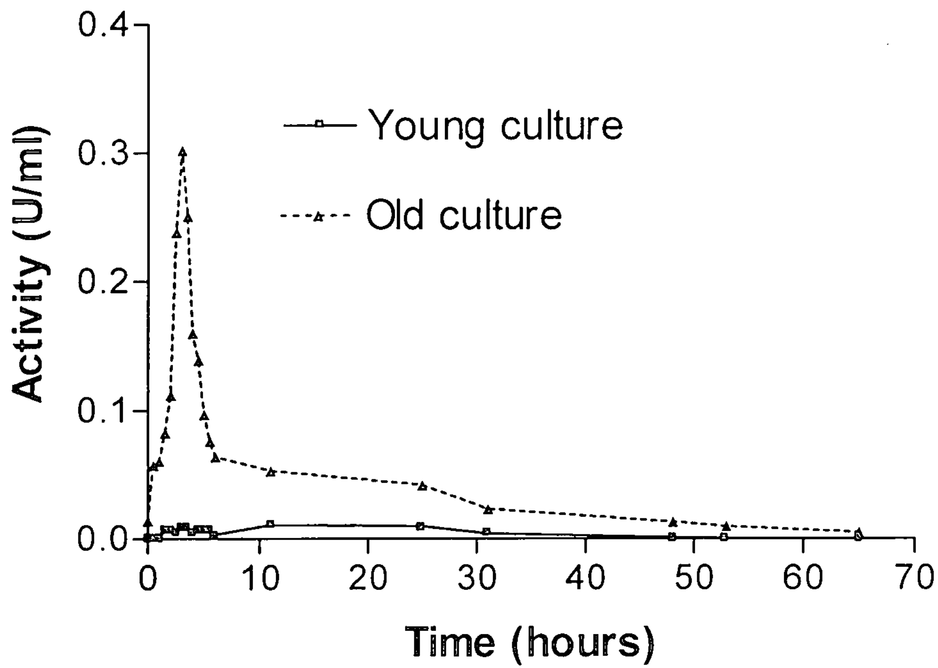



**Figure 4.3:** **Time-dependent lipase production after the cells were grown to their maximum before induction.**

(ii) The second experiment was carried out to compare the lipase production of a young culture with that of an older culture that already produced lipase on agar plates (Figure 4.4). In the case of both *E. dermatiditis* UOFS Y-2044 and *E. dermatiditis* UOFS Y-2048, maximal lipase production was observed with the older culture. The young culture produced negligible amounts of lipase activity.

(a)




(b)

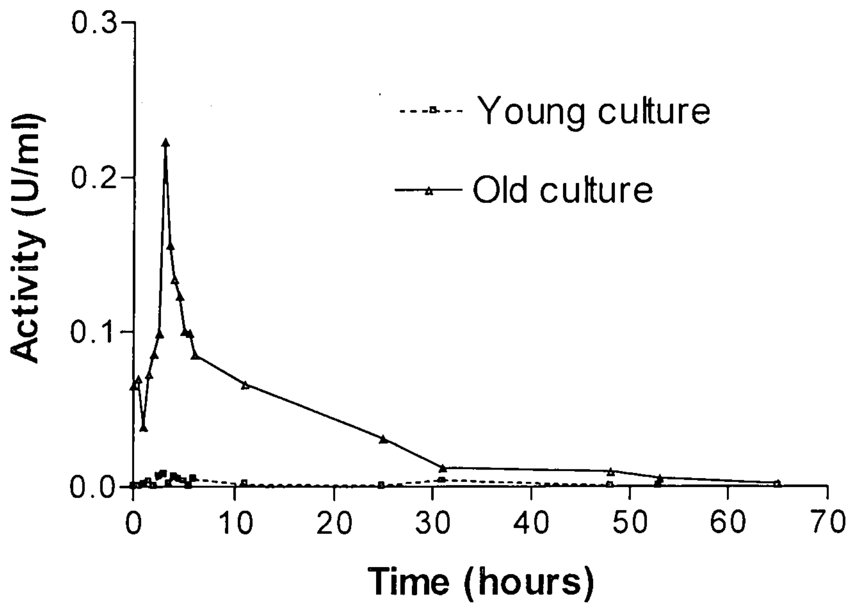



Figure 4.4: **Time-dependent lipase production of a young culture compared with an older culture. (a) Profile for ED2044L, (b) Profile for ED2048L.**

(iii) The third experiment was carried out to compare lipase production in two different media, namely (a) water and olive oil and (b) the standard YM-media with olive oil as inducer. The water and olive oil medium resulted in low lipase production (Figure 4.5) when compared with the standard YM-media (Figure 4.2).

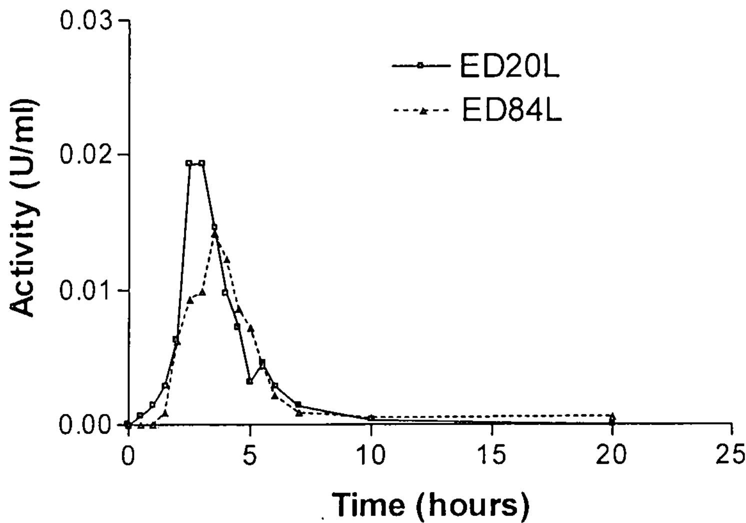
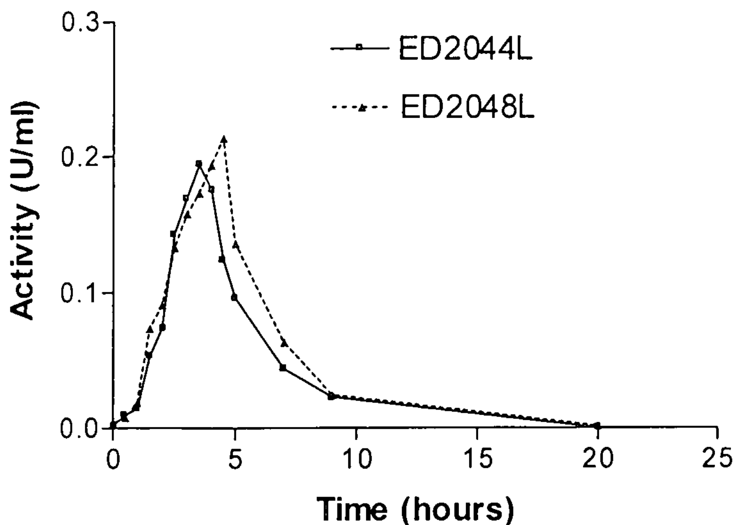



**Figure 4.5: Time-dependent lipase production in water and olive oil medium.**

(iv) The fourth experiment was carried out to compare lipase production after inoculating using a whole plate and after inoculating using a loop. It is clear from Figure 4.6 that inoculation using the whole plate results in the highest lipase production. The activity of the enzymes when inoculating using a loop was almost 50% less.

(a)



(b)

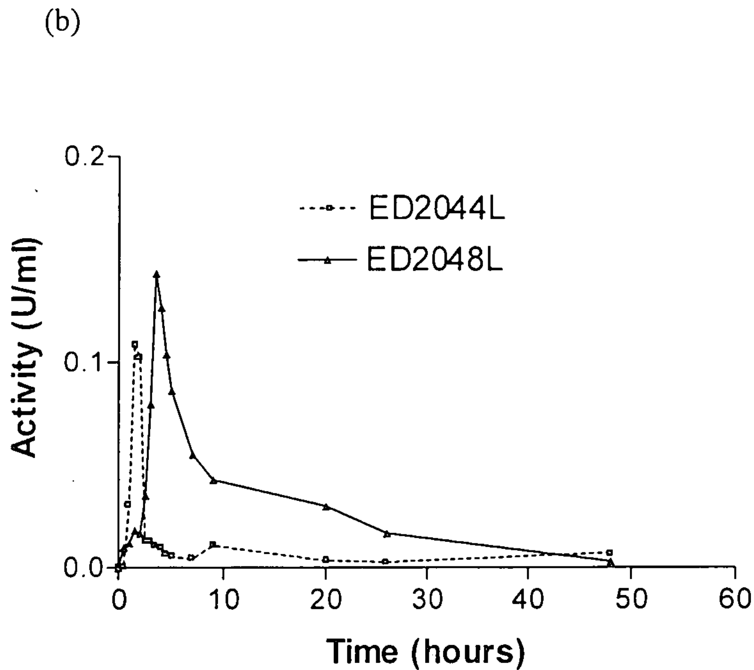



**Figure 4.6:** **Time-dependent lipase production when inoculating using a whole plate compared with inoculating using a loop. (a) a whole plate and (b) using a loop.**

## 4.2 Purification of *Exophiala dermatiditis* UOFS Y-2044 lipase

A wide range of fractionation methods and chromatography media were implemented in the purification of this lipase as well as in the purification of *E. dermatiditis* UOFS Y-2048 lipase (section 4.3).

### 4.2.1 Assessment of binding to MIMETIC A6XL dye adsorbent ligands using the Piksi kit

Lipase production in liquid culture peaked after only three hours. The culture was centrifuged and the supernatant was treated with 1mM PMSF to inhibit protease activity. An experiment was done first to determine the effect of PMSF on the enzyme. PMSF was added to a final concentration of 1mM to the crude enzyme before any purification steps were done. Aliquots were taken three times a day and assayed using the *p*NPP assay. The crude enzyme was stored at 4°C. After seven weeks the enzyme had retained about 70% of its initial activity.

The first chromatography step, size exclusion chromatography (SEC) on Toyopearl HW50F, was mainly used for a buffer exchange, although a degree of purification was obtained. This resulted in a broad $A_{280nm}$ peak with two smaller activity peaks (Figure 4.7). A yield of 319% and a

purification factor of almost 10 were obtained (Table 4.4). The pooled active fractions were used to assess binding on the Piksi kit (Table 4.2), which resulted in almost no enzyme binding.

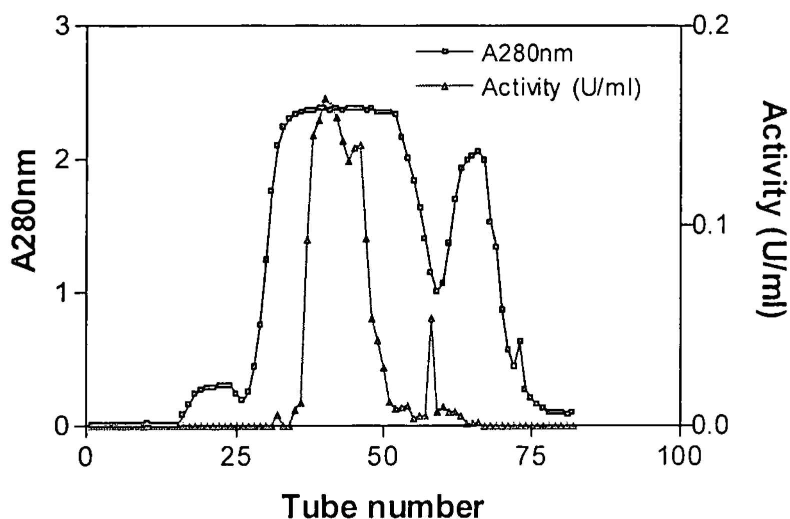



Figure 4.7: **Elution profile of SEC on Toyopearl HW50F (0.1M TRIS-HCL buffer, pH8,5)**

Table 4.2: **Yields of ED2044L bound and not bound to MIMETIC A6XL dye adsorbent ligands (0,1M TRIS-HCl, pH8,5). 0,414Units applied to each 1ml column.**

| Column | Not bound (%) | Bound (%) |
|---|---|---|
| 1 Red-1 | 27,5 | 2,1 |
| 2 Red-2 | 99,6 | 2,2 |
| 3 Orange-1 | 143,7 | 5,8 |
| 4 Orange-2 | 45,1 | 0 |
| 5 Orange-3 | 40,1 | 6,0 |
| 6 Yellow-1 | 88,6 | 1,2 |
| 7 Yellow-2 | 189,1 | 7,0 |
| 8 Green-1 | 81,2 | 19,3 |
| 9 Blue-1 | 82,9 | 7,3 |
| 10 Blue-2 | 89,5 | 5,6 |

A lipase sample was again loaded onto a Toyopearl HW50F SEC column under the same conditions except the buffer used was different. A 0,025M sodium phosphate buffer, pH6,0, was used in an attempt to improve binding of the lipase to the MIMETIC A6XL dye adsorbent dye ligands (Piksi kit) This buffer change resulted in a sharper absorbance peak with higher activity (Figure 4.8). The active fractions were pooled and used to assess binding on the Piksi kit. Better results were obtained (Table 4.3). Since MIMETIC Red-2 and MIMETIC Yellow-1 bound the most lipase activity it was decided to purchase 50ml of each of these two affinity chromatography media for further assessment.

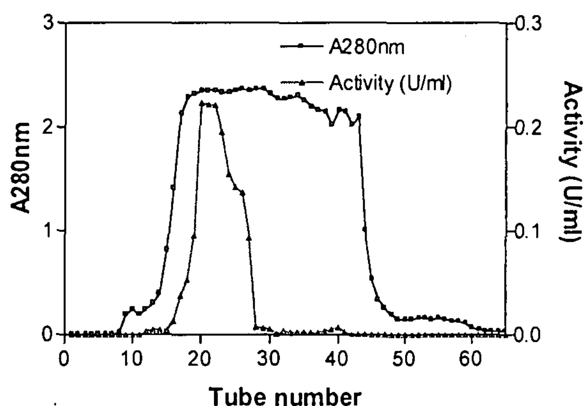



Figure 4.8: Elution profile of SEC on Toyopearl HW50F (0,025M phosphate buffer, pH6,0).

Table 4.3: **Yields of ED2044L bound and not bound to MIMETIC A6XL dye adsorbent ligands (0,025M phosphate buffer, pH6,0). 0,3372Units was applied to each 1ml column..**

| Column | Not bound (%) | Bound (%) |
|---|---|---|
| 1 Red-1 | 70,5 | 45,5 |
| 2 Red-2 | 87,9 | 47,6 |
| 3 Orange-1 | 0 | 47,9 |
| 4 Orange-2 | 0 | 40,5 |
| 5 Orange-3 | 0 | 42,9 |
| 6 Yellow-1 | 181,7 | 46,4 |
| 7 Yellow-2 | 83,9 | 20,6 |
| 8 Green-1 | 67,9 | 20,9 |
| 9 Blue-1 | 52,6 | 23,1 |
| 10 Blue-2 | 67,6 | 23,3 |

### 4.2.2 First isolation attempt

A decision was taken to decrease the concentration of the phosphate buffer to 0,01M resulting in an even sharper activity peak on the Toyopearl HW50F SEC column (Figure 4.9). The active fractions were pooled and applied to the MIMETIC Yellow-1 A6XL dye adsorbent ligand column (Figure 4.10). A yield of 38.35% was obtained with a purification factor of almost two (Table 4.).

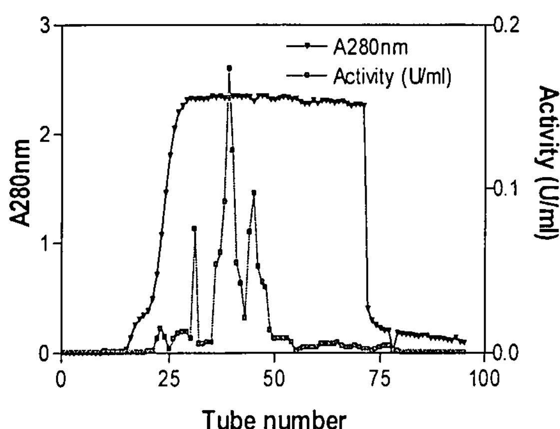



Figure 4.9: **Elution profile of SEC on Toyopearl HW50F (0,01M phosphate buffer, pH5,8).**

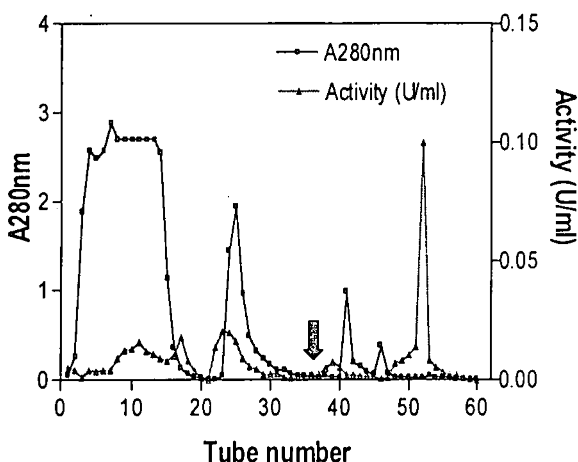



Figure 4.10: **Elution profile of affinity chromatography on MIMETIC Yellow-1 A6XL dye adsorbent ligand (0,01M phosphate buffer, pH5,8). The arrow indicates the start of the gradient.**

### 4.2.3 Second isolation attempt

Crude enzyme was loaded onto a Toyopearl HW50F SEC column using a 0,01M phosphate buffer, pH5,8 (Figure 4.9). After this an increase in yield and activity was obtained (Table 4.4), possibly as a result of removal of an inhibitor. The active fractions were pooled and loaded onto the MIMETIC Red-2 A6XL column previously equilibrated with a 0,01M phosphate buffer, pH 5,8. A sharp activity peak with high activity was obtained (Figure 4.11). A lower yield was obtained than with the MIMETIC Yellow-1 A6XL column, but a much higher specific activity and a purification factor

of almost 179 was obtained. The MIMETIC Red-2 A6XL column was then chosen for further purification attempts.

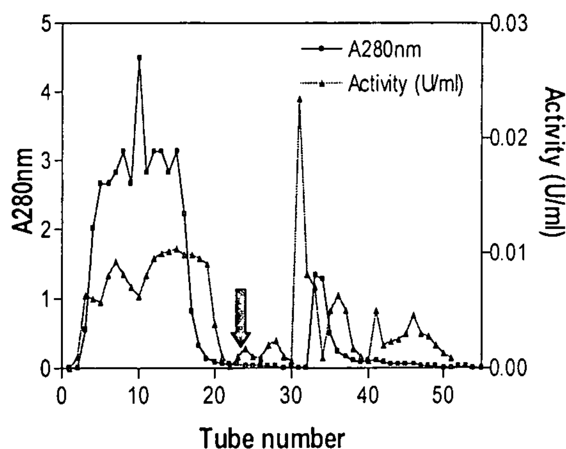



Figure 4.11: **Elution profile of affinity chromatography on MIMETIC Red-2 A6XL dye adsorbent ligand (0,01M phosphate buffer, pH5,8). The arrow indicates the start of the KCl-gradient.**

### 4.2.4 Third isolation attempt

As the lipase was not pure after the two-step purification protocol of SEC followed by affinity chromatography (data not shown), it was decided to assess ethanol precipitation followed by ion exchange chromatography (IEC) on Toyopearl DEAE-650M as possible additional purification steps. The yield after ethanol precipitation was 65,5%. This active fraction was loaded onto a Toyopearl DEAE-650M column previously equilibrated in a 0,01M TRIS-HCl buffer, pH8,0. The enzyme was eluted using a 0-2M KCl gradient (Figure 4.12).

Almost no enzyme bound to the column. A yield of 1,6% and a purification factor of almost one were obtained. 71% of the enzyme did not bind to the column (Table 4.4).

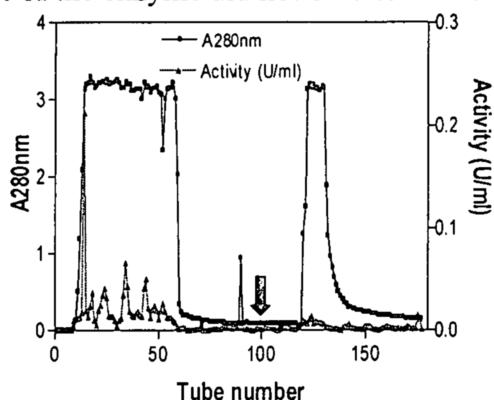



Figure 4.12: **Elution profile of IEC on Toyopearl DEAE-650M (0,01M TRIS-HCl buffer, pH8,0). The arrow indicates the start of the KCl-gradient.**

### 4.2.5 Fourth isolation attempt

Crude enzyme was loaded onto a Toyopearl SP-650M column previously equilibrated in a 0,05M phosphate buffer, pH5,8 (Figure 4.13). No enzyme bound to the column, resulting in a purification factor of 2,7 (Table 4.4).

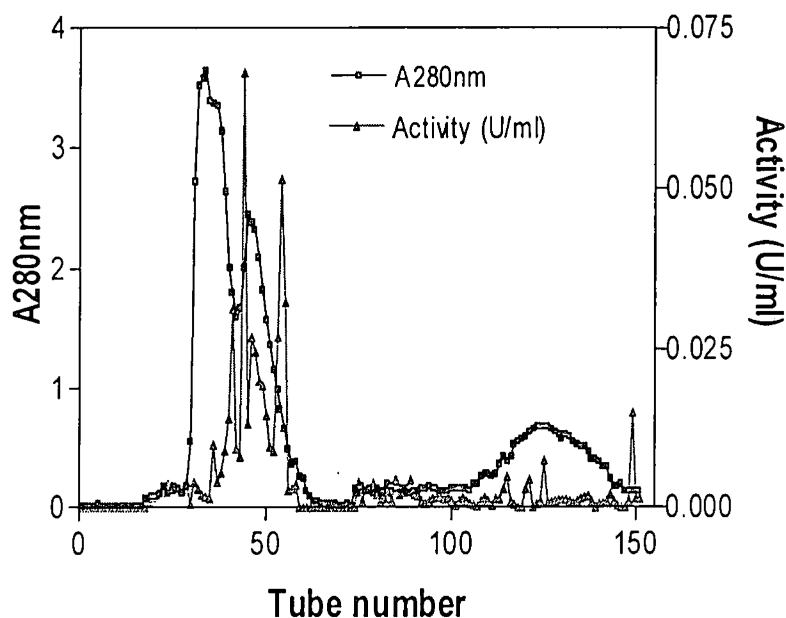



Figure 4.13: Elution profile of IEC on Toyopearl SP-650M (0,05M phosphate buffer, pH5,8).

### 4.2.6 Fifth isolation attempt

Crude enzyme was loaded onto a Toyopearl Super-Q 650S column previously equilibrated in a 0,05M phosphate buffer, pH8,0 (Figure 4.14). Almost no enzyme bound to the column and a purification factor of 2,8 was obtained (Table 4.4).

### 4.2.7 Sixth isolation attempt

Crude enzyme was loaded onto the Toyopearl SP-650M column, previously equilibrated in a 0,05M phosphate buffer, pH5,8 and eluted (Figure 4.13). Although no enzyme bound to this column, this step was necessary for binding of the enzyme to the next column, namely the Toyopearl Super-Q column. The active fractions obtained after IEC on the SP-column were pooled and loaded onto the Super-Q column and eluted using a 0-2M KCl gradient (Figure 4.14). This resulted in a sharp activity peak with a yield of 104,1% and a purification factor of 114,5. From

these results it was concluded that a compound in the cultivation medium was inhibiting lipase activity as well as binding of lipase to the Super-Q column. This compound is probably being removed by IEC on the Toyopearl SP-650M column thus facilitating binding of the lipase to the Super-Q column.

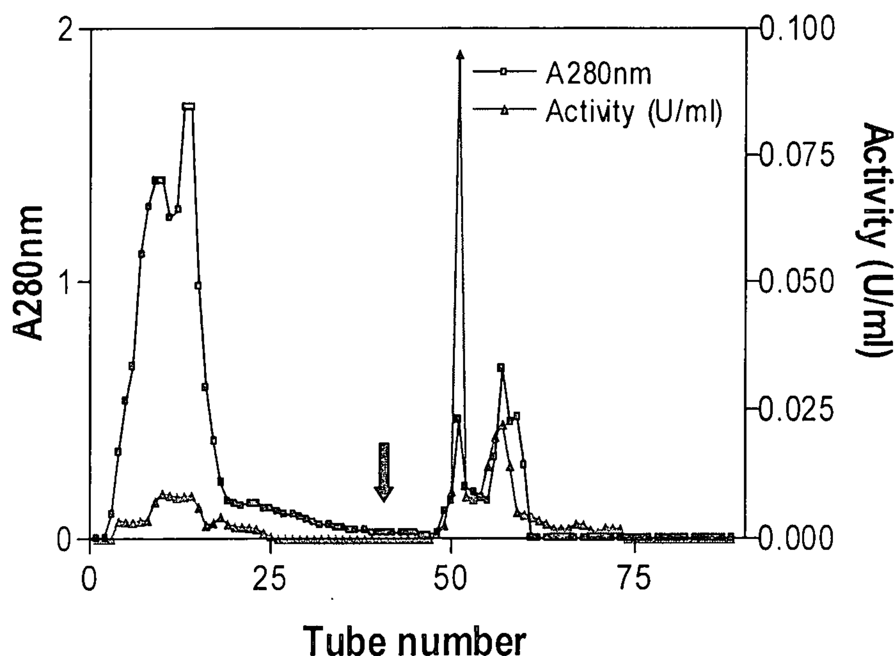



Figure 4.14: **Elution profile of IEC on Toyopearl Super-Q 650S (0,05M phosphate buffer, pH8,0). The arrow indicates the start of the KCl-gradient.**

### 4.2.8 Seventh isolation attempt

The final purification protocol for ED2044L consisted of four chromatography steps, namely: (a) IEC on Toyopearl SP-650M (0,05M phosphate buffer. PH5,8) (Figure 4.13); (b) IEC on Toyopearl Super-Q 650S (0,05M phosphate buffer, pH8,0) (Figure 4.14); (c) SEC on Toyopearl HW50F (0,01M phosphate buffer, pH5,8) (Figure 4.9) and (d) affinity chromatography on MIMETIC Red-2 A6Xl (0,01M phosphate buffer, pH5,8) (Figure 4.11). A final yield of 42,7% and a purification factor of 57,7 were obtained (Table 4.4). SDS-PAGE revealed that this purification protocol resulted in pure enzyme (Figure 4.20).

Table 4.4: Purification table of ED2044L

| Attempt | Step | Volume (ml) | Activity (U/ml) | Total activity (U) | Yield (%) | [Protein] (mg/ml) | Specific activity (U/mg) | Purification factor |
|---|---|---|---|---|---|---|---|---|
| 1 | **Culture supernatant** | 40,0 | 0,091 | 3,6 | 100 | 3,23 | 0,03 | 1 |
| | SEC (0.01M phosphate buffer. pH5.8) | 43,5 | 0,088 | 3,8 | 105,5 | 2,93 | 0,03 | 1,1 |
| | **MIMETIC Yellow** | | | | | | | |
| | Bound | 20,7 | 0,067 | 1,4 | 38,4 | 0,06 | 1,11 | 39,6 |
| | Not bound | 10,0 | 0,044 | 0,4 | 12,1 | 0,23 | 0,20 | 7 |
| 2 | **Culture supernatant** | 40,0 | 0,091 | 3,6 | 100 | 3,23 | 0,03 | 1 |
| | **SEC** | 43,5 | 0,088 | 3,8 | 105,5 | 0,43 | 0,21 | 7,4 |
| | **MIMETIC Red** | | | | | | | |
| | Bound | 18,9 | 0,072 | 1,4 | 37,6 | 0,01 | 5,00 | 178,6 |
| | Not bound | 15,0 | 0,015 | 0,2 | 6,2 | 0,21 | 0,07 | 2,6 |
| 3 | **Culture supernatant** | 200,0 | 0,096 | 19,9 | 100 | 3,25 | 0,03 | 1 |
| | **Ethanol precipitation** | 46,0 | 0,015 | 0,7 | 3,4 | 0,66 | 0,02 | 0,8 |
| | **Toyopearl DEAE-650M** | | | | | | | |
| | Bound | 41,2 | 0,008 | 0,3 | 1,6 | 0,35 | 0,02 | 0,7 |
| 4 | **Culture supernatant** | 40,0 | 0,062 | 2,5 | 100 | 3,23 | 0,02 | 1 |
| | **Toyopearl SP-650M** | | | | | | | |
| | Not bound | 48,5 | 0,018 | 0,9 | 34,5 | 0,34 | 0,05 | 2,7 |
| 5 | **Culture supernatant** | 35,0 | 0,062 | 2,1 | 100 | 3,23 | 0,02 | 1 |
| | **Toyopearl Super-Q 650S** | | | | | | | |
| | Not bound | 45,5 | 0,021 | 0,9 | 43,8 | 0,41 | 0,05 | 2,7 |
| 6 | **Culture supernatant** | 40,0 | 0,062 | 2,5 | 100 | 3,23 | 0,02 | 1 |
| | **Toyopearl SP-650M** | 48,5 | 0,018 | 0,9 | 34,5 | 0,34 | 0,05 | 2,7 |
| | **Toyopearl Super-Q 650S** | | | | | | | |
| | Bound | 53,5 | 0,048 | 2,6 | 301,4 | 0,02 | 2,17 | 114,5 |
| | Not bound | 54,0 | 0,033 | 1,8 | 222,9 | 0,14 | 0,23 | 12 |
| 7 | **Culture supernatant** | 100,0 | 0,117 | 11,7 | 100 | 3,75 | 0,03 | 1 |
| | **Toyopearl SP-650M** | 160,0 | 0,098 | 15,7 | 134,2 | 0,78 | 0,13 | 4,3 |
| | **Toyopearl Super-Q 650S** | 60,0 | 0,098 | 5,9 | 50,4 | 0,04 | 0,26 | 8,7 |
| | **SEC** | 60,0 | 0,114 | 6,8 | 58,1 | 0,07 | 1,63 | 54,3 |
| | **MIMETIC Red** | 52,0 | 0,087 | 5,0 | 42,7 | 0,56 | 1,73 | 57,7 |

## 4.3 Purification of *E. dermatiditis* UOFS Y-2048 lipase

### 4.3.1 Assessment of binding to MIMETIC A6XL dye adsorbent ligands using the Piksi kit

ED2048 was cultured with olive oil as lipase inducer to a point of maximum lipase production. The supernatant obtained after centrifugation contained the crude lipase and 200ml of this was loaded onto a Toyopearl HW50F SEC column pre-equilibrated with a 0,01M phosphate buffer, pH5,8. The resultant elution profile is depicted in Figure 4.15.

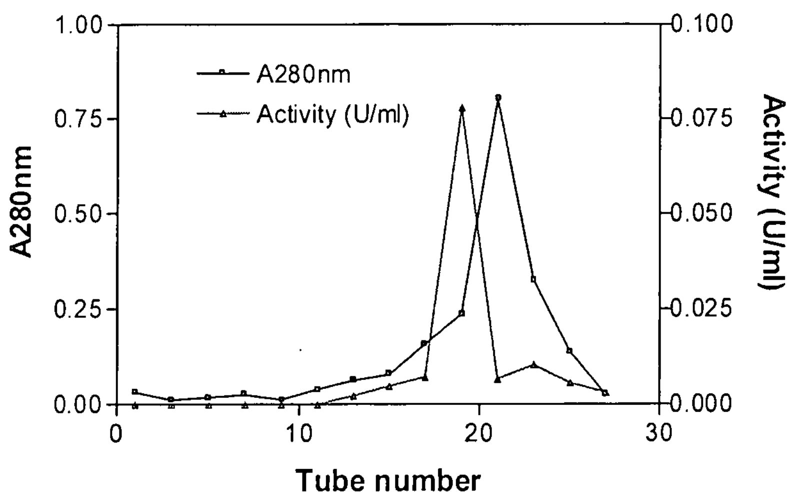



**Figure 4.15:** **Elution profile of SEC on Toyopearl HW50F (0,01M phosphate buffer, pH5,8).**

The active fractions from the SEC step were pooled and loaded onto a MIMETIC Yellow-1 A6XL column pre-equilibrated with a 0,01M phosphate buffer, pH5,8. After washing, lipase activity was eluted using a 0-2M KCl gradient (Figure 4.16). A purification factor of 1010,8 and a yield of 39,2% was obtained (Table 4.5).

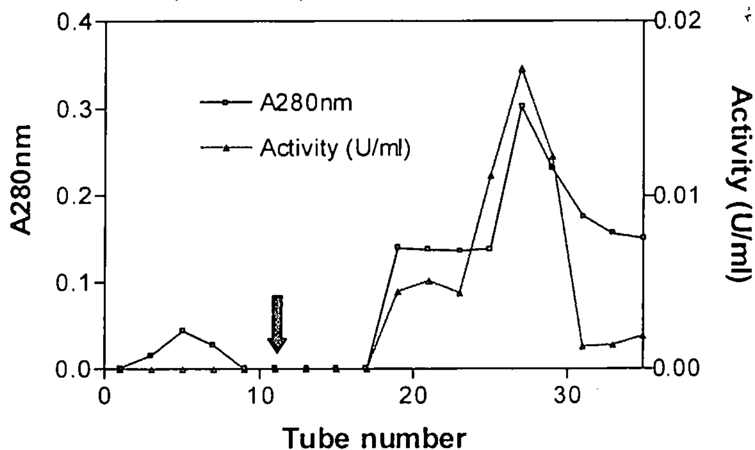



**Figure 4.16:** **Elution profile of affinity chromatography on MIMETIC Yellow-1 A6XL affinity ligand (0,01M phosphate buffer. pH5,8). The arrow indicates the start of the salt gradient.**

The active fractions obtained after SEC on Toyopearl HW50F were pooled and used to assess binding on the MIMETIC Red-2 A6XL dye adsorbent ligand column (0,01M phosphate buffer, pH5,8) (Figure 4.17). A lower purification factor of 96,7 as well as a lower yield of 0,5% was obtained. A large percentage of lipase activity (6,1%) did not bind to the MIMETIC Red-2 A6XL dye adsorbent ligand column. When the two affinity chromatography steps were compared, it became evident that the MIMETIC Yellow-1 A6XL dye adsorbent ligand was a better option for the purification of ED2048L.

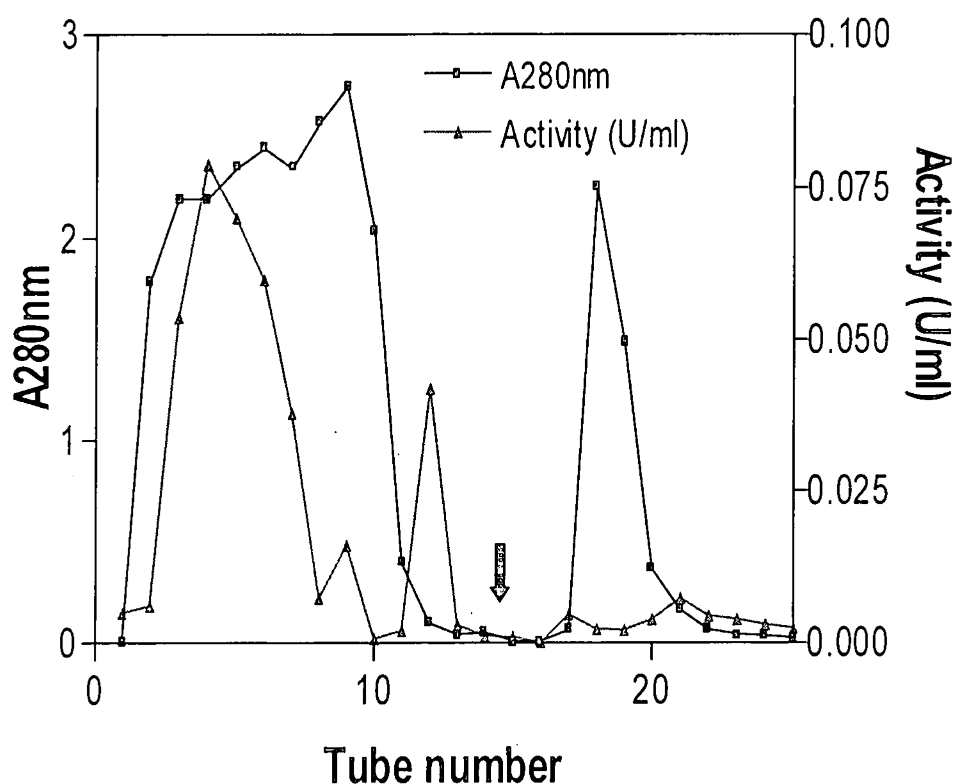



**Figure 4.17: Elution profile of affinity chromatography on MIMETIC Red-2 A6XL affinity ligand (0,01M phosphate buffer, pH5,8). The arrow indicates the start of the salt gradient.**

### 4.3.2 Purification protocol

The purification protocol used for ED2048L was the same as that used for ED2044L except for the last column step where MIMETIC Yellow-1 A6XL dye adsorbent ligand (Figure 4.16) was substituted for MIMETIC Red-2 A6XL dye adsorbent ligand (Figure 4.17). Figures 4.18 and 4.19 show the elution profiles of IEC on Toyopearl SP-650M and IEC on Toyopearl Super-Q 650S, respectively. This protocol resulted in a hopefully pure enzyme. A low purification factor of three and a specific activity of 1,6 was obtained (Table 4.5). The protein concentration was lower than that of ED2044L which led to problems being experienced with SDS-PAGE. An approximate

molecular mass could not be determined as no bands were visible on the SDS-PAGE gel even after silver staining. Given more time, this problem may have been resolved.

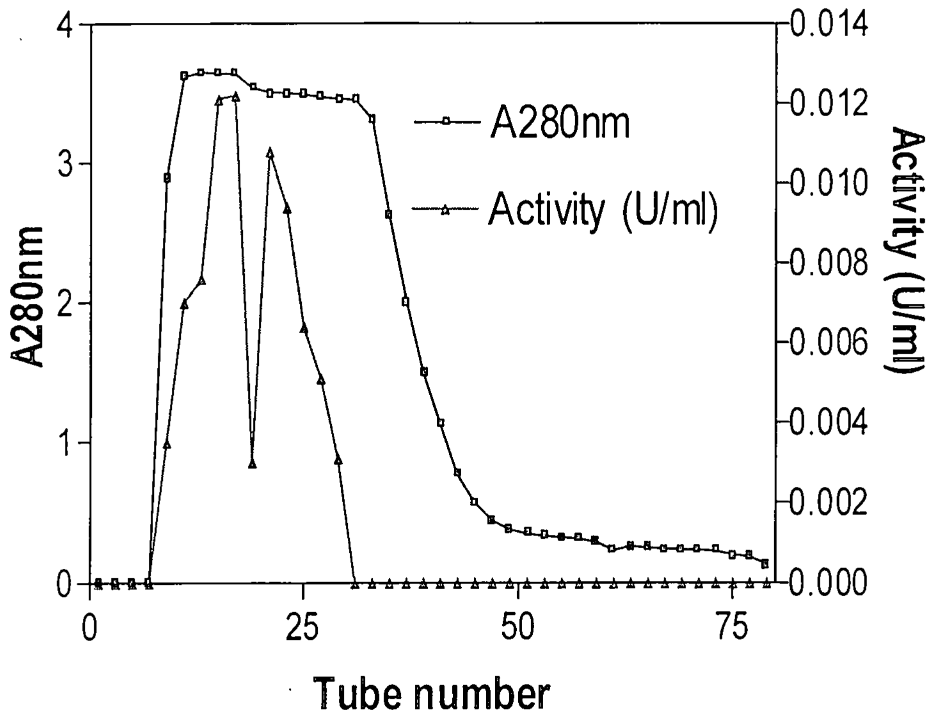



Figure 4.18 Elution profile of IEC on Toyopearl SP-650M (0,05M phosphate buffer, pH5,8).

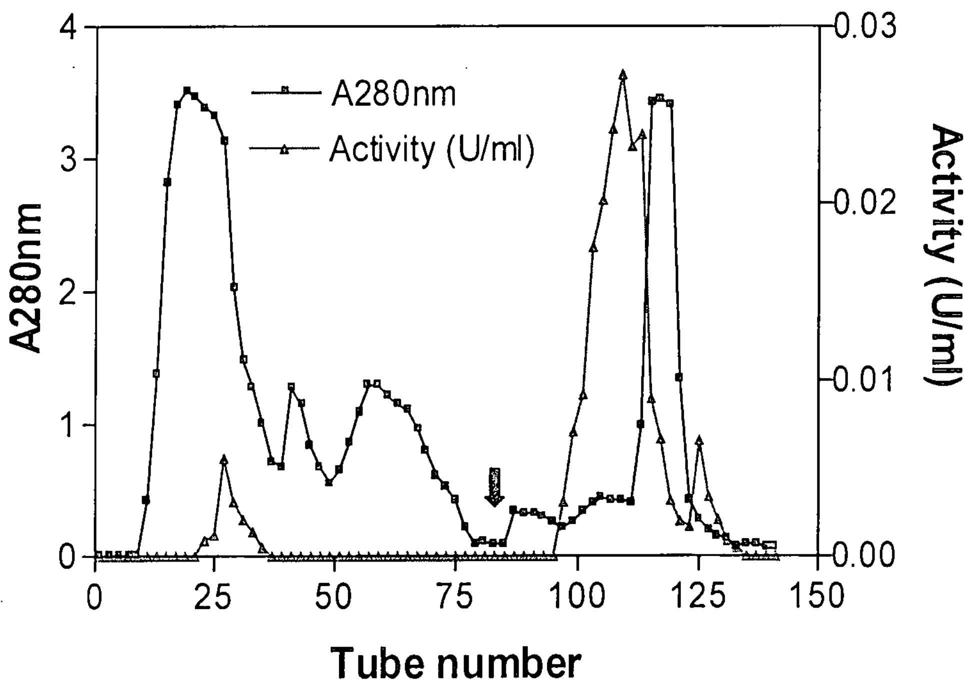



Figure 4.19 Elution profile of IEC on Toyopearl Super-Q 650S (0,05M phosphate buffer, pH5,8). The arrow indicates the start of the gradient.

Table 4.5: Purification table of ED2048L

| Attempt | Step | Volume (ml) | Activity (U/ml) | Total activity (U) | Yield (%) | [Protein] (mg/ml) | Specific activity (U/mg) | Purification factor |
|---|---|---|---|---|---|---|---|---|
| 1 | **Culture supernatant** | 200,0 | 0,050 | 9,9 | 100 | 4,03 | 0,01 | 1 |
| | **SEC** (0.01M phosphate buffer, pH5.8) | 48,8 | 0,047 | 2,3 | 23,1 | 3,90 | 0,01 | 1 |
| | **MIMETIC Yellow Bound** | 46,0 | 0,085 | 3,9 | 39,2 | 0,01 | 12,1 | 1010,8 |
| | **Not bound** | 41,2 | 0,067 | 2,8 | 28,3 | 2,13 | 0,03 | 3 |
| 2 | **Culture supernatant** | 200,0 | 0,050 | 9,9 | 100 | 4,00 | 0,01 | 1 |
| | **SEC** | 48,8 | 0,047 | 2,3 | 23,1 | 3,90 | 0,01 | 1 |
| | **MIMETIC Red Bound** | 9,0 | 0,006 | 0,1 | 0,5 | 0,01 | 1,16 | 96,7 |
| | **Not bound** | 16,7 | 0,038 | 0,6 | 6,1 | 2.35 | 0.02 | 2 |
| 3 | **Culture supernatant** | 200,0 | 0,018 | 3,6 | 100,0 | 6,70 | 0,54 | 1 |
| | **Toyopearl SP-650M** | 75,5 | 0,017 | 1,3 | 35,1 | 4,30 | 0,29 | 0,5 |
| | **Toyopearl Super-Q 650S** | 61,0 | 0,004 | 0,3 | 7,2 | 0,11 | 2,36 | 4,4 |
| | **SEC** | 37,5 | 0,008 | 0,3 | 8,0 | 0,02 | 12,61 | 23,4 |
| | **MIMETIC Yellow** | 61,5 | 0,008 | 0,5 | 13,5 | 0,01 | 1,62 | 3,0 |

## 4.4 Characterisation of lipases

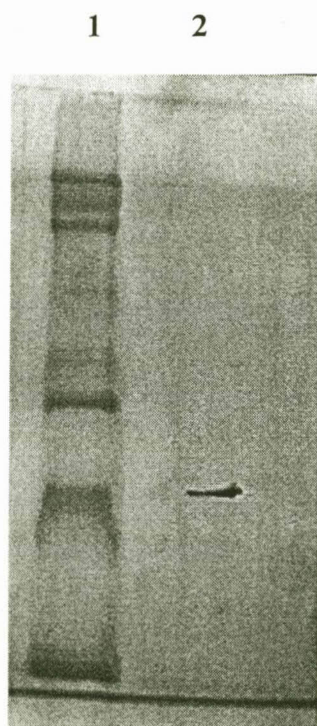
The lipase of *Exophiala dermatiditis* UOFS Y-2044 was characterised with respect to its molecular mass, thermostability, optimum temperature and pH, substrate specificity, interfacial activation, pH stability, the effect of metal ions and detergents and the effect of EDTA and PMSF. The purified enzyme fraction from the seventh isolation was stored at 4°C and used for characterisation.

The lipase of *Exophiala dermatiditis* UOFS Y-2048 was characterised with respect to its thermostability, optimum temperature and pH, pH stability, substrate specificity and interfacial activation. The enzyme fraction from the third isolation was stored at 4°C and used for characterisation.

### 4.4.1 Molecular mass determination

The isolation procedure described in section 4.2.8 resulted in the isolation of an pure enzyme with a relative molecular mass ($M_r$) of approximately 23 600 on SDS-PAGE (Figure 4.20). Figure 4.21 shows the calibration curve used to determine the approximate molecular mass of ED2044L.

1 2



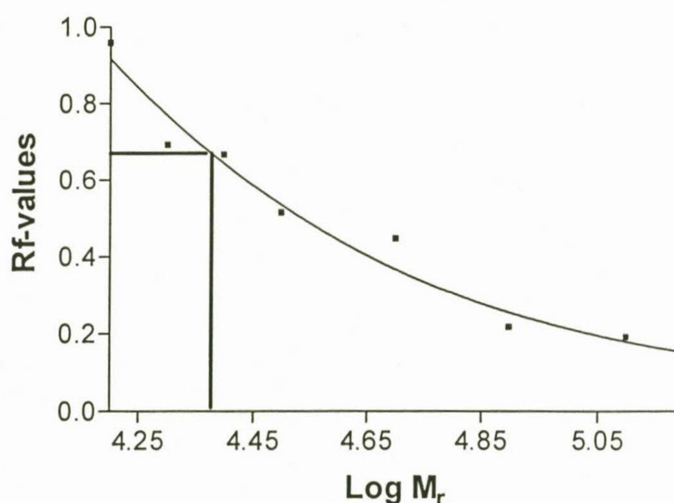



**Figure 4.21: Calibration curve of Rf-values vs. Log ($M_r$) used to determine the approximate $M_r$ of ED2044L.**

**Figure 4.20: SDS-PAGE gel of purified ED2044L. Lane 1 contains the marker proteins (listed in section 3.2.3.1); Lane 2 shows the isolated lipase.**

The molecular mass of ED2044L compared well with those of lipases from *Bacillus* sp.. The purified lipase of lipases from *Bacillus subtilis* showed a $M_r$ of 23 000 whereas an unidentified *Bacillus* sp. Lipase had a $M_r$ of 22 000 (Lessuisse *et al.*, 1993; Möller *et al.*, 1991). The lipases produced by *Bacillus licheniformis* and *Bacillus pumilus* both had a $M_r$ of 20 000 (Nthangeni, 1997). Nishio *et al.* (1987) reported a relative molecular mass of 33 000 for *Pseudomonas fragi* 22.39B lipase. According to Iizumi *et al.* (1990) the *Pseudomonas* sp. KWI-56 lipase exhibited a molecular mass of 38 000. The yeast lipase produced by *Candida deformans* CBS2071 exhibited an $M_r$ of 207 000 (Muderhwa *et al.*, 1985). Three lipases have been isolated from *Candida*, Lipases A, B and C, with molecular masses of 362 000, 200 000 and 143 000, respectively (Shaw *et al.*, 1989). A $M_r$ of 86 000 was reported for hormone-sensitive lipase isolated from chicken adipose tissue (Anthonsen *et al.*, 1997).

### 4.4.2 Thermostability

The thermostabilities of ED2044L and ED2048L at temperatures between 20°C and 80°C are depicted in Figures 4.22 and 4.23, respectively. Both the enzymes were stable at lower temperatures, but unstable at temperatures higher than 35°C. Approximate half-lives were determined for both the enzymes and are tabulated in Table 4.6. ED2048L was more stable at higher temperatures than ED2044L

Both the enzymes are not thermostable when compared to other enzymes. Khyami-Horani *et al.* (1996), reported a half-life of 30 min at 70°C for the lipase of *Bacillus licheniformis*. *Bacillus pumilus* lipase lost 20% of its activity after 30 minutes at 40°C, and almost 90% of its activity after incubation at 50°C (Nthangeni, 1997). *Rhizopus japonicus* NR 400 lipase retained 50% of its activity after 30 minutes at 55°C (Suzuki *et al.*, 1988).

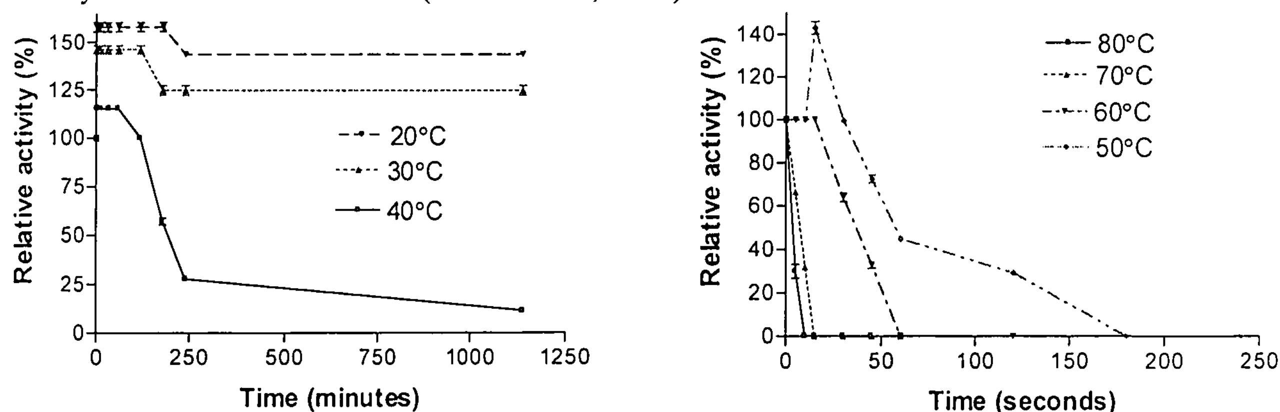



**Figure 4.22: Thermostability of ED2044L at different temperatures. Standard deviations are shown as error bars (n=3).**

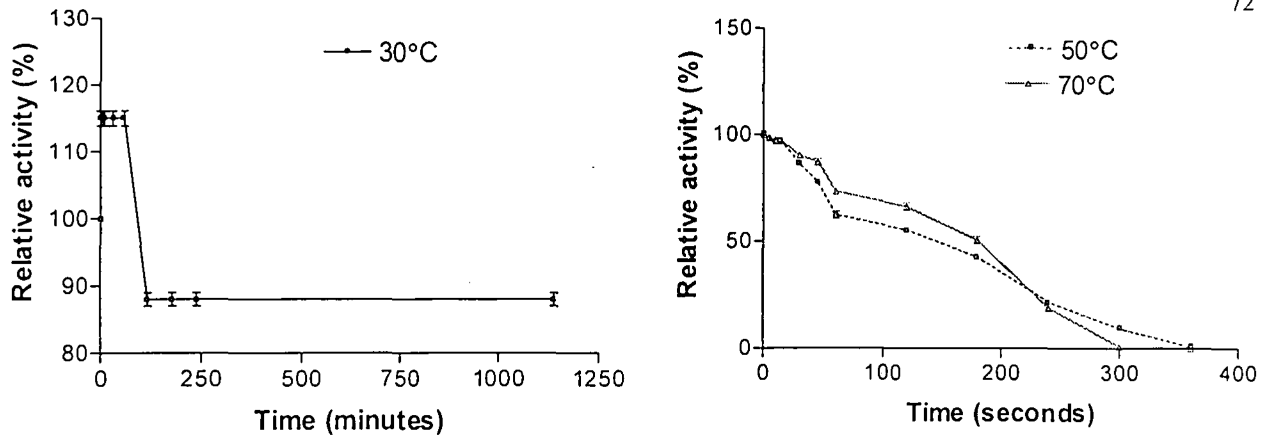



Figure 4.23: **Thermostability of ED2048L at different temperatures. Standard deviations are shown as error bars (n=3).**

Table 4.6: **Thermostability of ED2044L and ED2048L.**

| Temperature (°C) | Approx. $T_{0.5}$ : ED2044L | Approx. $T_{0.5}$ : ED2048L |
|---|---|---|
| 20 | >24h | |
| 30 | >24h | >24h |
| 40 | 3,2h | |
| 50 | 28 seconds | 2,9 minutes |
| 60 | 15 seconds | |
| 70 | 9,5 seconds | 3,2 minutes |
| 80 | 3,5 seconds | |

### 4.4.3 Optimum temperature

Figure 4.24 shows the optimum temperatures of ED2044L and ED2048L. ED2044L and ED2048L exhibited maximal activity at 50°C and 65°C, respectively. The maximum activities were normalized to 100%.

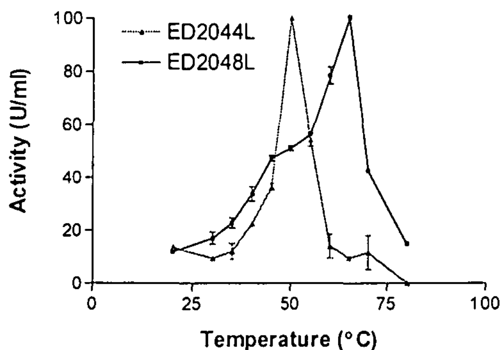



Figure 4.24: **Optimum temperature of ED2044L and ED2048L. Standard deviations are shown as error bars. (n=3)**

These temperature maxima are higher than those reported for the *Candida deformans* (Zach) Langeron and Guerra lipases of between 40°C and 50°C (Muderhwa *et al.*, 1985). The lipase

produced by *Aspergillus niger* showed optimum activity at 25°C (Hatzinikolaou *et al.*, 1996). According to Wu *et al.* (1996) the optimum temperature of the *Rhizomucor miehei* lipase is 50°C.

### 4.4.4 Optimum pH

The optimum pHs of ED2044L and ED2048L are shown in Figure 4.25. The activity at pH8,5 was taken as 100%. Both the lipases were active under alkaline conditions with an optimum pH of 8,5 at 37°C. The stability of the assay mixture decreased at higher pH values and became totally unstable at pH values higher than pH11,0.

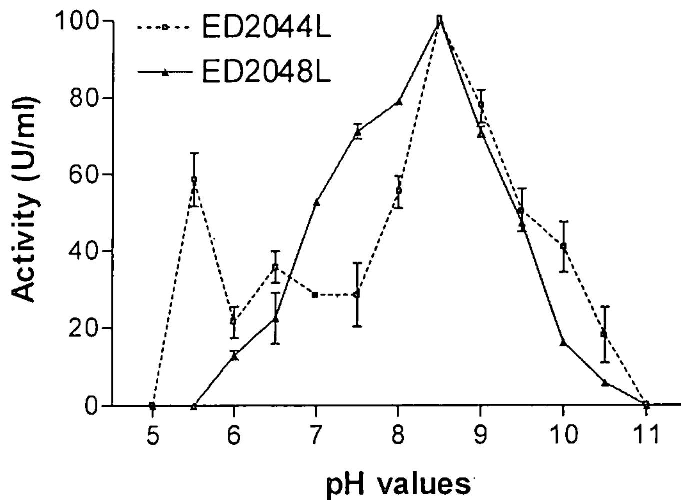



**Figure 4.25: Optimum pH of ED2044L and ED2048L. Standard deviations are shown as error bars. (n=3)**

In general, the alkaline pH optima of ED2044L and ED2048L are higher than the pH optima reported for fungal and yeast lipases. Maximal lipase activity of *Rhizomucor miehei* lipase was observed at pH7,0 (Wu *et al.*, 1996). *Geotrichum candidum* produced two lipases, namely Lipase I and II with optimal pH's of pH8,0 and 6,0 for the two lipases, respectively (Sugihara *et al.*, 1990). Lipase I and II of *Candida rugosa* had a broad pH optima range from 5,8 to 6,8 (Veeraragavan and Gibbs, 1989).

### 4.4.5 pH stability

Activity at pH8,0 was taken as 100% as most of the purification studies were done at this pH (figure 4.26). ED2044L retained full activity when incubated at pH7,0 and pH8,0, whereas ED2048L retained full activity when incubated at pH8,0 and pH9,0. Shifting the pH from these values resulted in loss of activity with less than 50% activity remaining after incubation at pH values 5 and 10. According to Hatzinikolaou *et al.* (1996) *Aspergillus niger* lipase was stable under alkaline

conditions, losing 50% of the initial activity after 2 and 55 minutes of incubation at pH8,0 and 9,0, respectively. At pH4-7 no significant differences in activity was observed. Maximum stability was obtained at pH8,0 with *Bacillus pumilus* lipase (Nthangeni, 1997). Möller *et al.* (1991) also reported alkaline stable lipases produced by their strains of *Bacillus pumilus*.

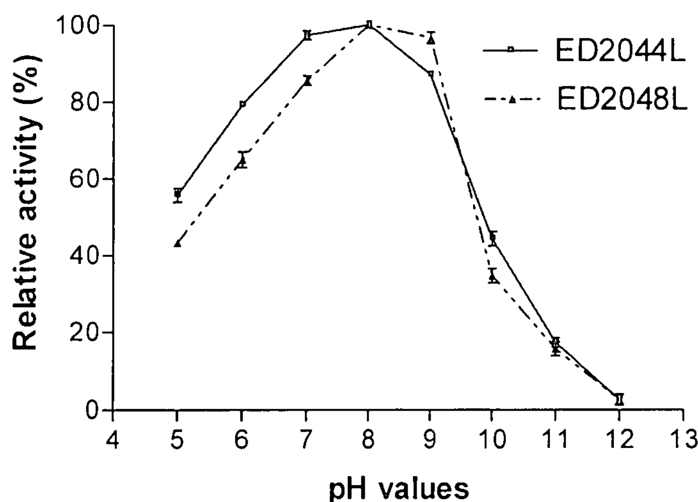



**Figure 4.26:** **pH stability of ED204L and ED2048L after incubation at 37°C for 30 minutes. Standard deviations are shown as error bars. (n=3)**

### 4.4.6 Substrate specificity

Both the lipases exhibited a wide specificity on the *p*-nitrophenyl esters (Figure 4.27a) as well as the triacylglycerols (Figure 4.27b) with a clear preference for esters of shorter chain fatty acids. Although the overall tendency of the two graphs is the same, some definite differences can be observed. ED2044L was more active on $C_3$ and $C_6$ *p*NP esters than ED2048L, whereas ED2048L was more active on $C_{14}$, $C_{16::0}$, $C_{18:2}$, and $C_{18:3}$ *p*NP esters than ED2044L. The activity of ED2048L on $C_3$ and $C_{18:1}$ triacylglycerols was 100-200% higher than that of ED2044L. These two lipases compare well with esterases and cutinases, which show maximum activity towards short chain fatty acids, which are relatively water soluble (Purdy and Kolattukudy, 1975; Köller and Kolattukudy, 1982). A lipase from *Aspergillus oryzae* showed high activities towards short chain fatty acid esters and was suggested to be a kind of esterase (Ohnishi *et al.*, 1994). *Geotrichum candidum* CMICC335426 Lipase B is very specific for hydrolysis of esters of cis-Δ9-fatty acids (Charton and Macrae, 1991). According to Muderhwa *et al.* (1986) the lipase of *Rhodotorula pilimanae* exhibits a preference for esters of longer chain fatty acids and an increase in the rate of hydrolysis of mono-unsaturated fatty acid chains as the chain lengths increased. The substrate preferences of ED2044L and ED2048L compare well with the lipase of *Candida deformans* CBS2071 (Muderhwa *et al.*,

1985) which exhibits a preference for esters of shorter chain fatty acids. *Humicola lanuginosa* lipase prefers medium chain fatty acids such as lauric acid (12:0), (Omar *et al.*, 1987).

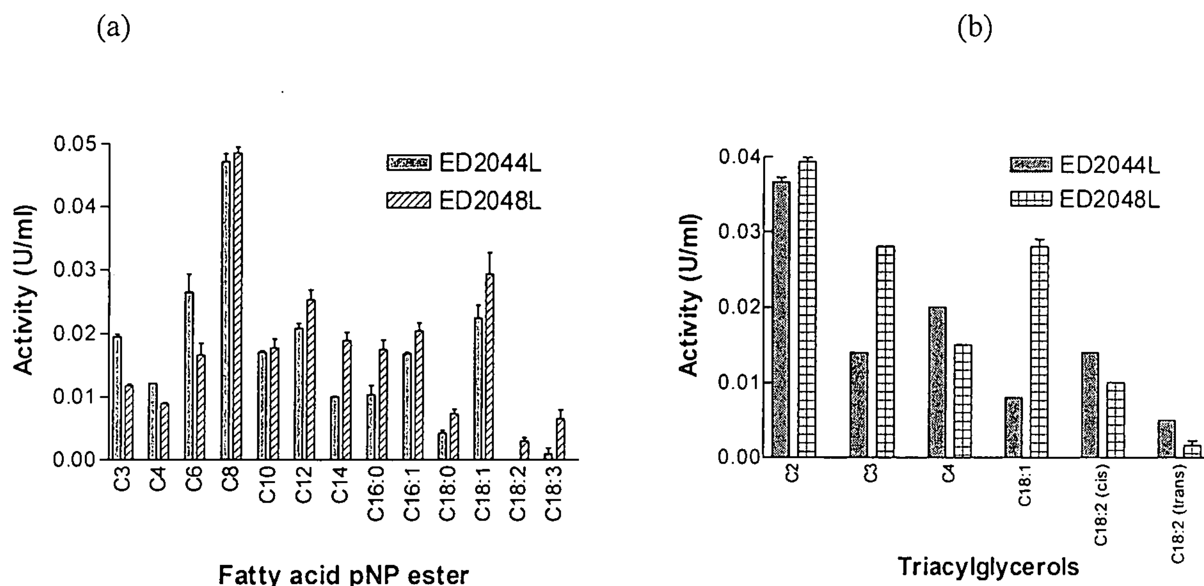



Figure 4.27: **Substrate specificities of ED2044L and ED2048L with (a) *p*-nitrophenyl esters and (b) triacylglycerols. Standard deviations are shown as error bars. (n=3)**

### 4.4.7 Interfacial activation

The effect of varying tripropionin concentrations on ED2044L and ED2048L activity is illustrated in Figure 4.28. The sigmoidal shape of the graphs indicates that both the lipases were activated by a lipid-water interface, a definition of "true" lipases. Below the critical micelle concentration of the substrate, the enzyme activity was zero, but once saturation of the substrate was exceeded, there was a sharp increase in enzyme activity. Most lipases that exhibit interfacial activation were found to possess a "lid", that covers the active center of the enzyme. Although the lid-based interfacial activation is still controversial, the results obtained suggested that there might be a lid covering the active site of these lipases. Most other lipases exhibit interfacial activation (Wooley and Petersen, 1994) except for a few exceptions such as the lipase of *Pseudomonas mendocina* (Bott *et al.*, 1993) and the cutinases (Derewenda *et al.*, 1994). Interfacial activation was reported for the human pancreatic lipase (Thirstrup *et al.*, 1994) and lipase B from *Candida cylindracea* (Rùa *et al.*, 1993). According to Stahmann *et al.* (1997) *Ashbya gossypii* lipase did not show interfacial activation with oleic acid as substrate.

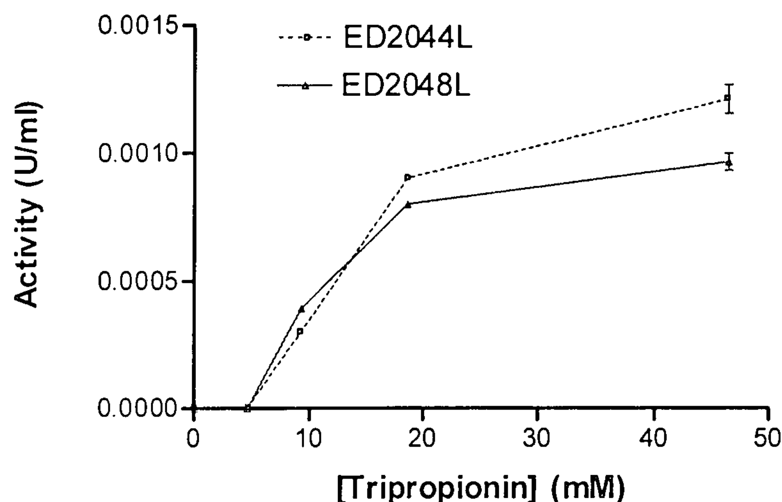



**Figure 4.28: Interfacial activation of ED2044L and ED2048L with tripropionin as substrate. Standard deviations are shown as error bars. (n=3)**

### 4.4.8 Effect of detergents

Lipases are water soluble, but they act on water insoluble substrates and could therefore be affected by detergents. The effects of different detergents on lipase activities are shown in Figure 4.29 (a-f). Due to time limitations, the effect of detergents was only tested on ED2044L.

Sodium deoxycholate activated the lipase activity below the cmc of 4mM and then inhibited it above the cmc (Figure 4.29 a). Sodium deoxycholate (5mM) is used in the assay mixture and from these results it might be concluded that only a slight under-estimation of the real lipase activity is observed because of the inhibition.

SDS (cmc = 8,2mM), an anionic detergent, inhibited ED2044L at all concentrations tested (Figure 4.29b) as did Cetrimide (cmc = 0,92mM), a cationic detergent (Figure 4.29c).

The non-ionic detergents, Triton-X-100 (cmc = 0,3mM) (Figure 4.29d) and Tween-80 (cmc = 0,01mM) (Figure 4.29e), activated ED2044L below the cmc of the detergent and inhibited lipase activity above the cmc. This activation has also been reported for *Chromobacterium viscosum* lipase which was activated by 0,25% Tween-80 (Helistö and Korpela, 1998). According to Helistö and Korpela (1998) lipases from *C. viscosum, Pseudomonas fluorescens* and *Bacillus* sp. Are also activated by Triton-X-100.

The zwitterionic detergent, CHAPS (cmc = 6,5mM), inhibited lipase activity at concentrations above and below the cmc (Figure 4.29f).

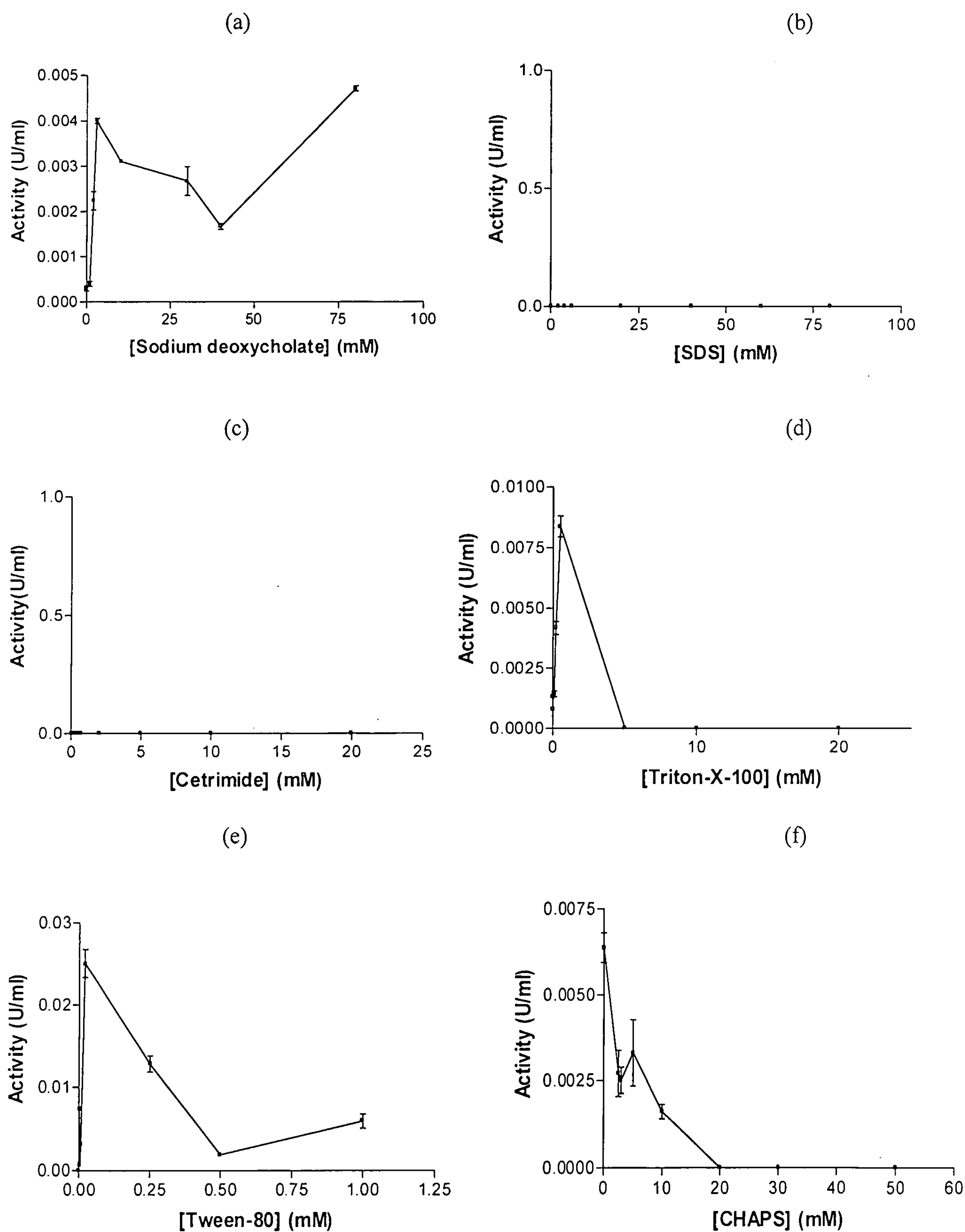
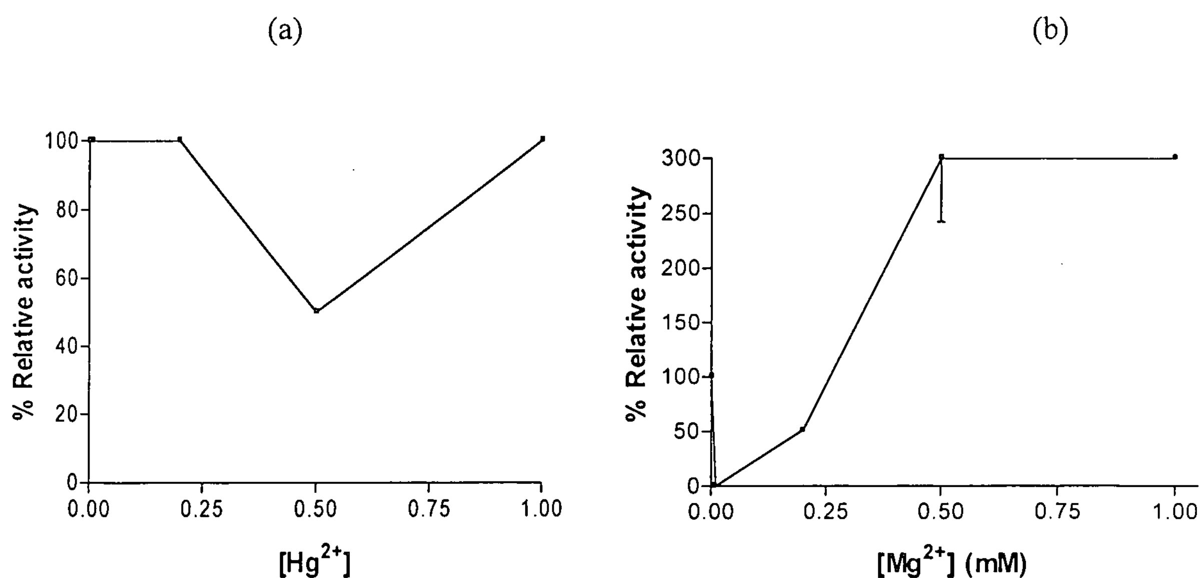



Figure 4.29: Effect of detergents on ED2044L activity. Standard deviations are shown as error bars. (n=3)

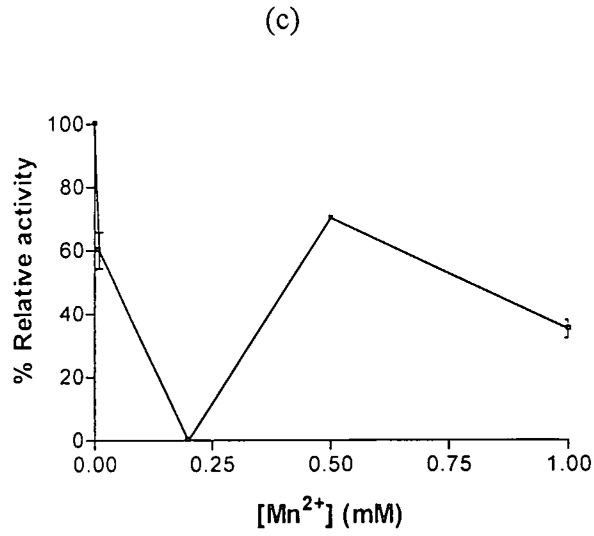
### 4.4.9 Effect of metal ions

The effect of metal ions on lipase activity is usually determined at a metal concentration of 1mM (Nishio *et al.*,1987; Omar *et al.*, 1987: Suzuki *et al.*, 1988), although 2mM, 3mM and 7mM concentrations were used by Hills and Beevers (1987), Muderhwa *et al.*, (1986) and Roussis *et al.* (1988), respectively. The effects of metal ions on ED2044L activity is shown in Figure 4.27(a-h). Metal ions tested in this study were: Manganese(II), Barium(II), Mercury(II), Iron(III), Magnesium(II), Copper(II), Calcium(II) and Tin(II). Only three of the eight metal ions had an inhibitory effect on ED2044L, namely calcium(II), tin(II) and copper(II). The other five metal ions activated the lipase activity.

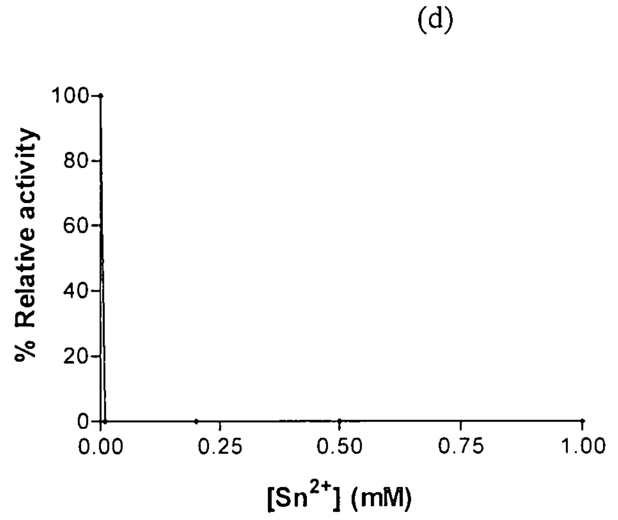
The following metal ions are known inhibitors of lipase: Ag(I) (Nishio *et al.*, 1987), Al(III) (Nishio *et al.,* 1987), Mn(II) (Muderhwa *et al.*, 1985), Sn(II) (Omar *et al.*, 1987) and Zn(II) (Nishio *et al.*, 1987; Muderhwa *et al.*, 1985). Ca(II) is usually known as a lipase activator (Hills and Beevers 1987). *Pseudomonas fragi* CRDA037 lipase is effectively inhibited by $Fe^{2+}$ and $Fe^{3+}$ (Schuepp *et al.*, 1997). *Aspergillus niger* lipase is also inhibited strongly by $Fe^{2+}$ and $Fe^{3+}$ (Schuepp *et al.*, 1997; Fukumoto *et al.*, 1963). According to Veeraragavan *et al.* (1990) the lipase produced by *Geotrichum candidum* is inhibited by $Ca^{2+}$, $Mg^{2+}$. $K^+$ and $Na^+$ had no effect on this lipase (Veeraragavan *et al.*, 1990).



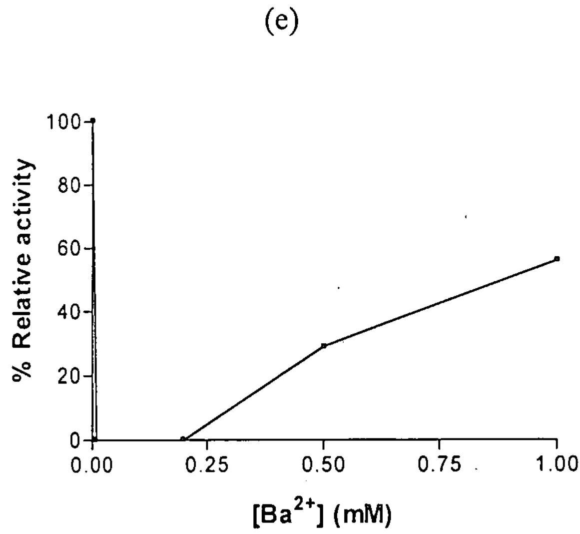
(c)



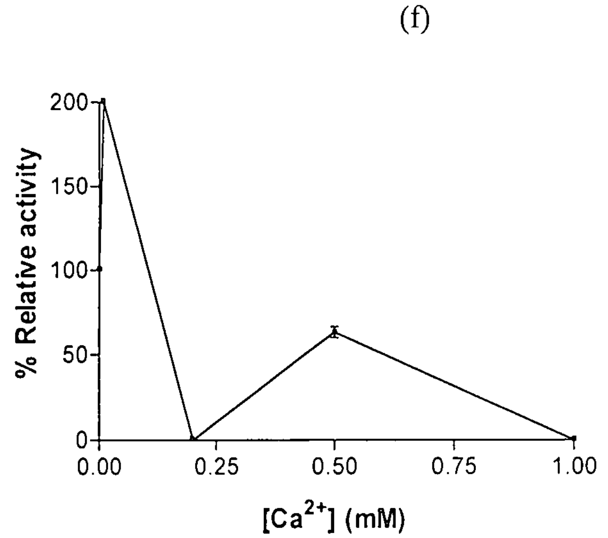

(d)



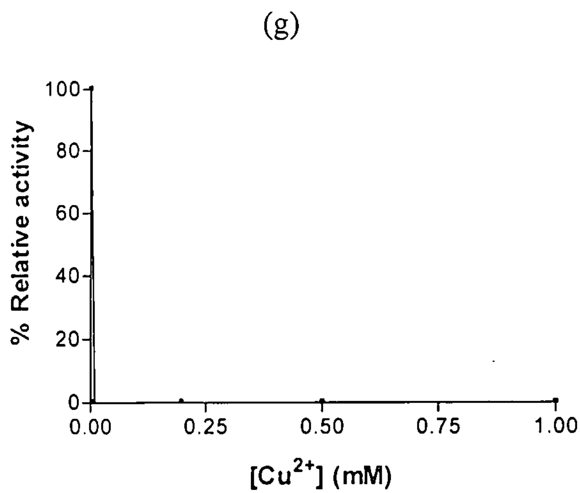

(e)




(f)




(g)




(h)

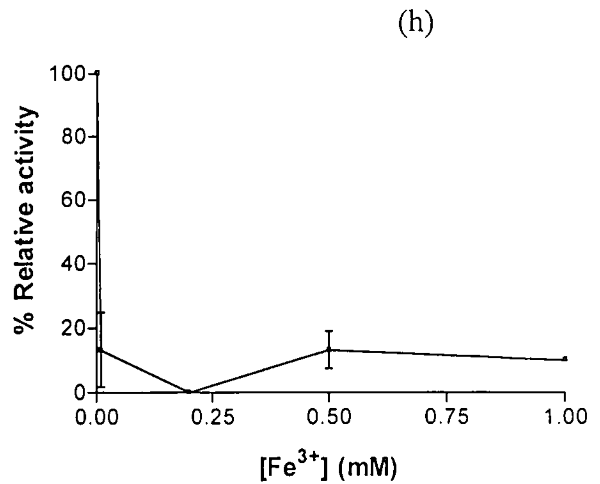



**Figure 4.30: Effect of metal ions on ED2044L activity. Standard deviations are shown as error bars.**

### 4.4.10 Effect of EDTA on lipase activity

The total inhibition of the ED2044L activity in the presence of EDTA (Figure 4.31) indicated that the lipase is dependent on a metal ion for activity. From these results it can be concluded that ED2044L is a metalloenzyme. The plant lipase isolated from castor bean lipid bodies (Hills and Beevers, 1987) is dependent on metal ions such as calcium(II) for its activity. *Bacillus pumilus* lipase activity was not affected by EDTA at low concentrations, whereas *Bacillus licheniformis* lipase is activated with 0,25mM EDTA. Higher concentrations of EDTA inhibited the lipases (Nthangeni, 1997). According to Labuschagne (1995) the lipase activity of *Flavobacterium odoratum* is activated (50%) by EDTA.

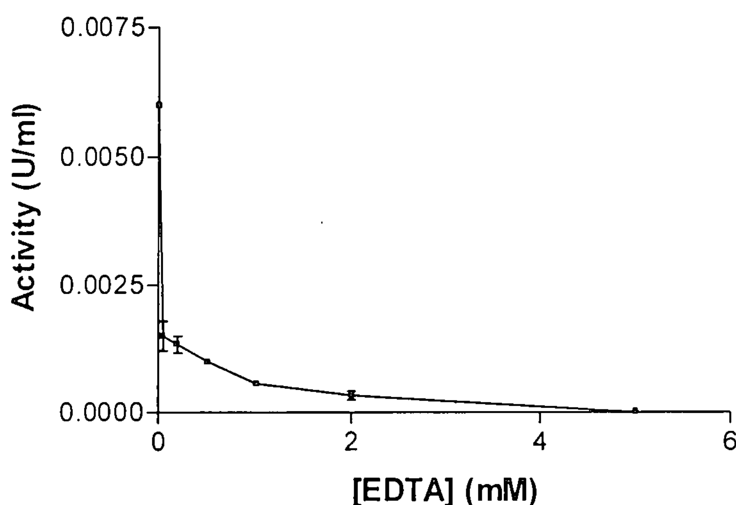



**Figure 4.31: Effect of EDTA on lipase activity. Standard deviations are shown as error bars.**

### 4.4.11 Effect of PMSF on lipase activity

The effect of PMSF on ED2044L is shown in Figure 4.33. These results are inconclusive as PMSF is a classical serine hydrolase inhibitor and only marginal inhibition was observed. A serine-containing pentapeptide sequence has been postulated to form part of the active site of lipases, with the serine residue playing an active role in catalysis, and it is this serine which is targeted by PMSF. Resistance to PMSF has been observed for other lipases and has been attributed to a protective burying of the active site within the enzyme by the "lid region" (Brady *et al.*, 1990). *F. odoratum* lipase is slightly inhibited by high concentrations of PMSF (Labuschagne, 1995). Both *Bacillus licheniformis* and *Bacillus pumilus* are inhibited by PMSF (Nthangeni, 1997).

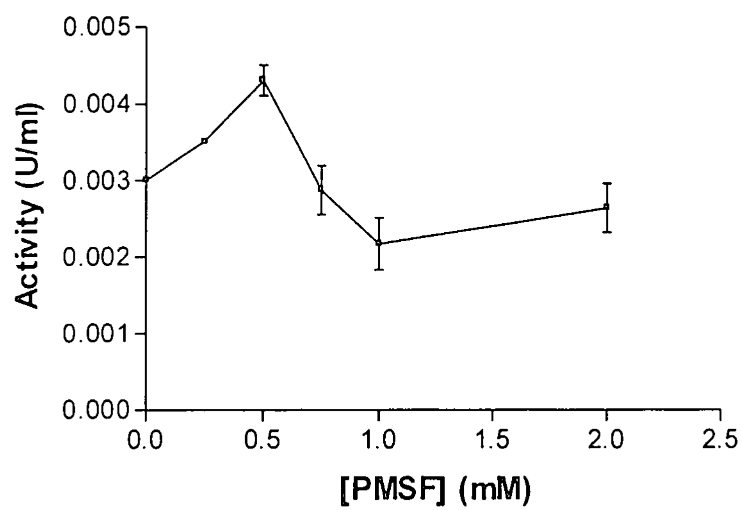



Figure 4.32: Effect of PMSF on ED2044L activity. Standard deviations are shown as error bars.

# CHAPTER 5

## GENERAL DISCUSSION AND CONCLUSIONS

Lipases are ubiquitous in nature, occurring in microorganisms, plant seeds and mammalian tissues, and it is thus not surprising that lipase activity was found in black yeasts. However, this study represents the first report of the isolation of a lipase from black yeasts and from the *Exophiala* species.

Yeast isolates were screened for lipase production on agar plates containing four different inducers. Isolates that showed lipase production on agar plates were grown in shake cultures containing yeast extract supplemented with olive oil as inducer. Two *Exophiala dermatiditis* strains, namely *Exophiala dermatiditis* UOFS Y-2044 and *Exophiala dermatiditis* UOFS Y-2048, were chosen from the isolates as best lipase producers, for further study. These two organisms secreted extracellular lipases. ED2048L activity was higher after cultivation and centrifugation than the activity of ED2044L. The lipase activity of both the enzymes peaked after only three to four hours, indicating that the secretion of the lipases are not dependent on cell growth.

Both the lipases were purified using chromatography steps. ED2044L was purified 58-fold with a 42,7% recovery from the culture supernatant of *E. dermatiditis* UOFS Y-2044. The molecular mass of ED2044L was estimated to be approximately 23 600. ED2048L was purified 3-fold with a recovery of 13,5% from the culture supernatant of *E. dermatiditis* UOFS Y-2048. Because of the low protein content of the purified ED2048L, no bands were visible on the SDS-PAGE gel even after silver staining. This protein may be glycosilated and this can interfere with the staining of the protein on a SDS-PAGE gel. ED2048L was purified for comparison with ED2044L.

The physical-chemical characterisation of the two lipases differed with only certain key characteristics being determined for ED2048L in order to compare it with ED2044L, which was more fully characterised. This was done to determine whether or not these two lipases are the same lipase produced by two different strains of the same organism.

Although both lipases were very similar to yeast and bacterial lipases reported in the literature, the conclusion was reached that ED2044L and ED2048L are in fact not the same enzyme and that the

two strains of *E. dermatiditis* differ sufficiently to produce lipases with their own distinct characteristics.

Both lipases were stable at alkaline pH values, but were not thermostable and are therefore not good candidates for biotechnological applications. The 3D structures of the two lipases are needed to understand the general occurrence and the importance of interfacial activation of the lid-like helical structures covering the active site. The relative high optimum temperature of ED2048L and ED2044L and their ability to be active under alkaline pH conditions, suggest that the lipases may be useful for the industry.

Analysis of the gene structure of both the lipases will provide useful information regarding the homology between ED2044L and ED2048L and related enzymes. After over expression of the lipase gene in a foreign host, secondary structure analysis could be used for 3D-structure modeling of the lipases. A more ambitious approach would be X-ray crystallographic studies on the lipases. Structural and modeling data will reveal more information about the catalytic triad and the presence of a "lid".

This study resulted in the conclusion that both the enzymes and the field of lipases in general are open for further investigation. As new crystal structures of lipases appear, it may become easier to see what is typical and atypical about lipases. Lipases remain a fascinating group of enzymes that have found many variations on a common theme in order to selectively break down all the types of ester bonds found in nature.

# REFERENCES

Alhir, S., Markakis, P. and Chandan, R.C. (1990). Lipase of *Penicillium casecolum*. *J. Agric. Food Chem.* **38**, p.598-601.

Anthonsen, M.W., Degerman, E. and Holm, C. (1997). Partial purification and identification of hormone-sensitive Lipase from chicken adipose tissue. *Biochem. Biophys. Research Comm.* **236**, p.94-99.

Babayan, V.K. (1987). Medium chain triacylglycerised and structured lipids. *Lipids* **22**, p.417-420.

Basri, M., Ampon, K., Zin Wan Yunus, W.M., Razak, C.N.A. and Bakar salleh, A. (1994). Immobilisation of hydrophobic lipase derivates onto organic polymer beads. *J. Chem. Technol. Biotechnol.* **59**, p.37-44.

Benzonana, G. and Esposito, S. (1971). On the positional and chain specificities of *Candida cylindracea* lipase. *Biochim. Biophys. Acta* **231**, p.15-22.

Berger, M., Laumen, K. and Schneider, M.P. (1992). Enzymatic esterification of glycerol. I. Lipase catalysed synthesis of regioisomerically pure 1,3-sn-diacylglycerols. *J. Am. Oil Soc.* **69**, p.955-960.

Berger, M. and Schneider, M.P. (1992). Enzymatic esterification of glycerol. II. Lipase catalysed synthesis of regioisomerically pure 1(3)-rac-monoacylglycerols. *J. Am. Oil Soc.* **69**, p.961-965.

Boel, E., Huge-Jensen, B., Wöldike, H.F., Gormsen, E., Christensen, M., Andreasen, F. and Thim, L. (1990). Cloning and expression of industrially important fungal lipases. In *Lipases: structure, mechanism and genetic engineering*. GBF monographs No. 16 (Alberghina, L., Schmid, R.D. and Verger, R., eds.), VCH, Weinheim, p.207-219.

Borzeix, F., Monot, F. and Vandecasteele, J-P. (1992). Strategies for enzymatic esterification in organic solvents: comparison of microaqueous, biphasic and micellar systems. *Enzyme Microb. Technol.* **14**, p.791-797.

Bosley, J. (1994). Applications of lipases in (inter)esterification. In Abstract book (*Characterization of lipases: for industrial applications: 3D structure and catalytic mechanism*), September 14-17, Bendor, France, 1994, p.62.

Bott, R., Wu, S., Dauberman, J., Power, S., Van Kimmenade, A., Jarnigan, A., Fallon, E., Boston, M., Paulose, A.J. and Sarma, R. (1993). The three-dimensional structure of a streamlined lipase. In

Abstract book *(Lipase: characterization, function and protein engineering*), October 10-13, Elsinore, Denmark, p.33.

Brady, L., Brzozowski, A.M., Derewenda, Z.S., Dodson, E., Dodson, G., Tolley, S., Turkenburg, J.P., Christiansen, L., Huge-Jensen, B., Norskov, L., Thim, L. and Menge, U. (1990). A serine protease triad forms the catalytic centre of a triacylglycerol lipase. *Nature* **343**, p.767-770.

Brzozowski, A.M., Derewenda, U., Derewenda, Z.S., Dodson, G.G., Lawson, D.M., Turkenburg, J.P., Bjorkling, F., Huge-Jensen, B., Patkar, S.A. and Thim, L. (1991). A model for interfacial activation in lipases from the structure of a fungal lipase-inhibitor complex. *Nature* **351**, p.491-494.

Charton, E. and Macrae, A.R. (1991). Substrate specificities of lipases A and B from *Geotrichum candidum* CMICC 335426. *Biochim. Biophys. Acta* **1123**, p.59-64.

Chung, G.H., Lee, Y.P., Yoo, O.J. and Rhee, J.S. (1991). Overexpression of a thermostable lipase gene from *Pseudomonas fluorescens in Escherichia coli. Appl. Microb. Biotechnol.* **35,** p.237-241.

Derewenda, Z.S., Wei, Y., Swenson, J.L., Schottel, S., Patkar, S. and Halkier, T. (1994). The crystal structure of a suberin degrading esterase from pathogenic *Streptomyces scables*. In Abstract book (*Characterization of lipase for industrial application: 3D structure and catalytic mechanism*), September 14-17, Bendor, France, p.24.

Derksen, J.T.P. and Cuperus, F.P. (1992). Lipase-catalysed hydrolysis of crambe oil in AOT-isooctane reversed micelles. *Biotechnol. Lett.* **14**, p.973-980.

Desnuelle, P. (1961). Pancreatic lipases. *Adv. Enzymology* **23**, p.129-161.

Duncombe, W.G. (1963). The colorimetric micro-determination of long-chain fatty acids. *Biochem. J.* **88**, p.7-10.

Dünhaupt, A., Lang, S. and Wagner, F. (1992). *Pseudomonas cepacia* lipase: studies on aggregation, purification and on the cleavage of olive oil. *Biotechnol. Lett.* **14**, p.953-958.

Finkelman, M.A.J. (1990). Yeast strain development for extracellular enzyme production. In *Yeast strain selection* (Pandral, C.J., ed.) Marcel Dekker, Inc., New York, Basel, p.185-213.

Fossati, P., Ponti, M., Paris, P., Berti, G. and Tarenghi, G. (1992). Kinetic colorimetric assay of lipase in serum. *Clin. Chem.* **38**, p.211-215

Fryer, T.F., Lawrence, R.C. and Reiter, B. (1966). Methods for isolation and enumeration of lipolytic organisms. *J. Dairy Sci.* **50**, p.477-484.

Fukumoto, J., Iwai, M. and Tsujisaka, Y. (1963). Studies on lipase. I. Purification and crystallisation of a lipase secreted by *Aspergillus niger Jour. Gen. Appl. Microb.* **9**, p.353-361.

Gilbert, E.J. (1993). *Pseudomonas* lipase: Biochemical properties and molecular cloning. *Enzyme Microb. Technol.* **15**. P.634-645.

Grochulski, P., Li, Y., Schrag, J.D., Bouthillier, F., Smith, P., Harrison, D., Rubin, B. and Cygler, M. (1993). Insights into the interfacial activation from an open structure of *Candida rugosa* lipase. *J. Biol. Chem.* **17**, p.12843-12847.

Hatzinikolaou, D.G., Macris, J.B., Christakopoulos, P., Kekos, D., Kolisis, F.N. and Fountaoukidis, G. (1996). Production and partial characterisation of extracellular lipase from *Aspergillus niger*. *Biotechnol. Lett.* **18**, p.547-552.

Helistö, P, and Korpela, T. (1998). Effects of detergents on activity of microbial lipases as measured by the nitrophenyl alkanoate esters method. *Enzyme Microb. Technol.* **23**, p.113-117.

Hendrickson, H.S. (1994). Fluorescence-based assays of lipases, phospholipases, and other lipolytic enzymes. *Anal. Biochem.* **219**, p.1-8.

Hills, M.J. and Beevers, H. (1987). $Ca^{2+}$ stimulated neutral lipase activity in castor bean lipid bodies. *Plant Physiol.* **84**, p.272-276.

Ignjatovic, M.W. and Dey, E.S. (1993). Characterisation of extracellular lipase from yeast. In Abstract book of (*Lipase: charcterization, function and protein engineering*), October 10-13, Elsinore, Denmark, p.55.

Iizumi, T., Nakamura, K. and Fukase, T. (1990). Purification and characterisation of a thermostable lipase from newly isolated *Pseudomonas* sp. *Agric. Biol. Chem.* **54**, p.1253-1258.

Jaeger, K.E., Ransac, S., Dijkstra, B.W., Colson, C., Van Heuvel, M. and Misset, O. (1994). Bacterial lipases. *FEMS Microb. Rev.* **15,** p.29-36.

Jensen, R.G., Galluzzo, D.R. and Bush, V.J. (1990). Selectivity is an important characteristic of lipases (acylglycerol hydrolases). *Biocatalysis* **3**, p.307-316.

Jette, J-F. and Ziomek, E. (1994). Determination of lipase activity by a Rhodamine-triglyceride-agarose assay. *Anal. Biochem.* **219**, p.256-260.

Khyami-Horani, H. (1996). Thermotolerant strain of *Bacillus licheniformis* producing lipase. *World. J. Microb. Biotechnol.* **12**, p.399-401.

Köller, W. and Kolattukudy, P.E. (1982). Mechanism of action of cutinase; chemical modification of the catalytic triad characteristic of serine hydrolases. *Biochemistry* **21**, p.3083-3090.

Kouker, G. and Jaeger, K-E. (1987). Specific and sensitive plate assay for bacterial lipases. *Appl. Environ. Microbiol.* **53**, p.211-213.

Kresse, H., Löffler, B-M. and Kunze, H. (1991). Improved procedure for the purification of hepatic lipase from rat liver homogenate. *Biochem. International* **23**,p.885-893.

Kurooka, S., Okamoto, S. and Hashimoto, M. (1977). A novel and simple colorimetric assay for human serum lipase. *J. Biochem.* **81**, p.361-369.

Labourdenne, S., Brass, O., Riviere, C., Verger, R., Cagna, A., Esposito, G. and Delorme, B. (1994). Automatic oil drop tensiometer for the measurement of (phospho)lipase kinetics at the triacylglycerol-water interface.

Labuschagne, R.B. (1995). *Flavobacterium odoratum* lipase: isolation and characterisation. M.Sc. dissertation, University of the Orange Free State.

Laemmli, U.K. (1970). Most commonly used discontinuous buffer system for SDS electrophoresis. *Nature* **227**, p.680-688.

Lessuisse, E., Schanck, K. and Colson, C. (1993). Purification and preliminary charcaterisation of the extracellular lipase of *Bacillus* subtitles 168, an extremely basic pH-tolerant enzyme. Eur. *J. Biochem.* **216**, p.155-160.

Lindsay, R.C. (1985) in *Food Chemistry* (Fennema, O.R., ed.) Marcel Dekker, New York, p.585-627.

Linfield, W.M., Barauskas, R.A., Sivieri, L., Serota, L. and Stevenson, R.W. (1984) *J. Am. Oil Chem. Soc.* **61**, p.191-195.

Macrae, A.R. and Hammond, R.C. (1985). Present and future applications of lipases. *Biotechnol. Gen. Eng. Rev.* **3**, p.193-217.

McConville, F.X., Lopez, J.L. and Wald, S.A. (1990) in *Biocatalysis* (Abtamowicz, D.A., ed.) van Nostrand Reinhold, New York, p.167-177.

McKellar, R.C. (1986). A rapid colorimetric assay for the extracellular lipase of *Pseudomonas fluorescens* B52 using β-naphthyl caprylate. *J. Diary Res.* **53**, p.117-127.

McNeill, G., Shimizu, S. and Yamane, T. (1990). Solid-phase enzymatic glycerolysis of beef tallow resulting in a high-yield of monoglyceride. *J. Am. Oil Chem. Soc.* **67**, p.779-783.

Mitsuda, S., Umemura, T. and Hirohara, H. (1988). Preparation of an optically pure secondary alcohol of synthetic pyrethroids using microbial lipases. *Appl. Microbiol. Biotechnol.* **29**, p.310-315.

Möller, B., Vetter, R., Wilke, D. and Foullois, B. (1991) Alkaline *Bacillus* lipase, coding DNA sequences therefore and *Bacilli* which produce the lipase. Patent no. W091/16422.

Muderhwa, J.M., Ratomahenina, R., Pina, M., Graille, J. and Galzy, P. (1985). Purification and properties of the lipase from *Candida deformans*. *JAOCS* **62**, p.1031-1036.

Nishio, T., Chikano, T. and Kamimura, M. (1987). Purification and some properties of lipase produced by *Pseudomonas fragi* 22.39B. *Agric. Biol. Chem.* **51**, p.181-186.

Norin, M., Edholm, O. and Hult, K. (1993). Modeling activation of lipases. In Abstract book (*Lipases: Structure, function and protein engineering*), October 10-13, Elsinore, Denmark, p.21.

Nthangeni, M.B. (1997). Partial purification and properties of two *Bacillus* lipases. M.Sc. dissertation, University of the Orange Free State.

Odera, M., Takeughi, K. and Toh-e, A. (1986). Molecular cloning of lipase genes from *Alkaligenes denitrificans* and their expression in *Escherichia coli*. *J. Ferment. Technol.* **64**, p.363-371.

Ohnishi, K., Joshida, Y., Toita, J. and Sekiguchi, J. (1994). Purification and characterisation of a novel lipolytic enzyme from *Aspergillus oryzae*. *J. Ferment. Bioeng.* **78**, p.413-419.

Olson, G.J., Woese, C.R. and Overbeek, R. (1994). The winds of evolutionary change: Breathing life into microbiology. *J. Bacteriol.* **176**, p.1-6.

Okumura, S., Iwai, M. and Tsujisaka, Y. (1980). Purification and properties of partial glyceride hydrolase of *Penicillium cyclopium*. *J. Biochem* **87**, p.205-211.

Okumura, S., Iwai, M. and Tsujisaka, Y. (1976). Positional specificities of four kinds of microbial lipases. *Agric. Biol. Chem.* **40**, p.655-660.

Omar, I.C., Hayashu, M. and Nagai, S. (1987). Purification and properties of a thermostable lipase from *Humicola lanuginosa.Agric. Biol. Chem.* **51**, p.37-45.

Ottolina, G., Carrea, G., Riva, S., Satore, L. and Veronese, F.M. (1992). Effect of the enzyme form on the activity, stability and enantioselectivity of lipoprotein lipase in toluene. *Biotechnol. Lett.* **14**, p.947-952.

Pedrocci-Fantoni, G. and Servi, S. (1992). Regio- and chemo-selective properties of lipase from *Candida cylindracea*. *J. Chem. Soc. Perken. Trans.* **1**, p.1029-1033.

Phillips, A. and Pretorius, G.H.J. (1991) Purification and characterisation of an extracellular lipase from *Galactomyces geotrichum*. *Biotechnol. Lett.* **13,** p.833-838.

Purdy, R.E. and Kolattukudly, P.E. (1975). Hydrolysis of plant cuticle by plant pathogens: Properties of cutinase I, cutinase II, and non-specific esterase isolated from *Fusarium solani pisi*. *Biochemistry* **14**, p.2832-2840.

Ransač, S., Rogalski, E., Gargouri, Y., Deveer, A.M.T.J., Paltauf, F., De Haas, G.H. and Verger, R. (1990). Stereoselectivity of lipases. I. Hydrolysis of enantiomeric glyceride analogues by gastric and pancreatic lipases. A kinetic study using the monolayer film technique. *J. Biol. Chem.* **265**, p.20263-20270.

Ransac, S., Riviere, C., Gancet, C., Verger, R. and De Haas, G.H. (1990). Competitive inhibition of lipolytic enzymes. I. A kinetic model applicable to water-insoluble competitive inhibitors. *Biochim. Biophys. Acta* **1043**, p.57-66.

Ransac, S., Gargouri, Y., Moreau, H. and Verger, R. (1991). Inactivation of pancreatic and gastric lipases by tetrahydrolipstatin and alkyl-dithio-5-(2-nitrobenzoic acid). A kinetic study with 1,2-didecanoyl-*sn*-glycerol monolayers. *Eur. J. Biochem.* **202,** P.395-400.

Rapp, P. and Backhaus, S. (1992). Formation of extracellular lipases by filamentous fungi, yeasts, and bacteria. *Enzyme Microb. Technol.* **14**, p.938-943.

Rawyler, A. and Siegenthaler, P.A. (1989). A single and continuous spectrophotometric assay for various lipolytic enzymes, using natural, non-labeled lipid substrates. *Biochim. Biophys. Acta* **1004**, p.337-344.

Rogalska, E., Ransac, S. and Verger, R. (1993). Controlling lipase stereoselectivity via the surface pressure. *J. Biol. Chem.* **268**, p.792-794.

Roussis, I.G., Karabalis, I., Papadopaulou, C. and Drainas, C. (1988). Some properties of extracellular lipase from a *Pseudomonas fluorescens* strain. *Lebensm., Wiss, Technol.* **21**, p.188-194.

Rùa, M.L., Diaz-Marino, T., Fernandez, V.M., Otera, C. and Ballesteros, A. (1993). Purification and characterisation of two distinct lipases from *Candida cylindracea*. *Biochim. Biophys. Acta* **1156**, p.181-189.

Safarik, I. (1991). A spectrophotometric assay for lipase activity utilising immobilised triacylglycerols. *J. Biochem. Biophys. Methods* **23**, p.249-253.

Samad, M.Y.A., Razak, C.N.A., Salleh, A.B., Zin Wan Yunus, W.M., Ampon, K. and Basri, M. (1989). A plate assay for primary screening of lipase activity. *J. Microbiol. Methods* **9,** p.51-56.

Sarda, L. and Desnuelle, P. (1958). Action de la pancreatique sur les esters and emulsion. *Biochim. Biophys. Acta* **30**, p.513-521.

Schmidt-Dannert, C., Sztajer, H., Stöcklein, W., Menge, U. and Schmid, R.D. (1994). Screening, purification and properties of a thermophilic lipase from *Bacillus thermocatenulatus*. *Biochim. Biophys. Acta* **1214**, p.43-53.

Shabtai, Y. and Daya-Mishne, N. (1992). Production, purification and properties of a lipase from a Bacterium (*Pseudomonas aeruginosa* YS-7). Capable of growing in water-restricted environments. *Appl. Environ. Microb.* **58**, p.174-180.

Shaw, J.F., Chang, C.H. and Wang, Y.J. (1989). Characterization of three distinct forms of lipolytic enzyme in a commercial Candida lipase preparation. Biotechnol. *Lett.* **11**, p.779-784.

Shimada, Y., Koga, C., Sugihara, A., Nagao, T., Takada, N., Tsunasawa, S. and Taminaga, Y. (1993). Purification and characterisation of a novel solvent-tolerant lipase from *Fusarium heterosporum*. *J. Ferment. Biotechnol.* **75**, p.349-352.

Smith, P.K., Krohn, R.U., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Otson, B.J. and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. *Anal. Biochem.* **150**, p.76-85.

Stahmann, K-P., Böddecker, T. and Sahm, H. (1997). Regulation and properties of a fungal lipase showing interfacial inactivation by gas bubbles, or droplets of lipid or fatty acid. *Eur. J. Biochem.* **244**, p.220-225.

Starace, C.A. (1983). Detergent enzymes – past, present and future. *Soap Cosmetics Chem. Spec.* **59**, p.48-50F.

Schuepp, C., Kermasha, S., Michalski, M-C. and Morin, A. (1997). Production, partial purification and characterisation of lipases from *Pseudomonas fragi* CRDA 037. *Proc. Biochem.* **32**, p.225-232.

Stuer, W., Jaeger, K-E. and Winkler, U.K. (1986). Purification of extracellular lipase from *Pseudomonas aeruginosa*. *J. Bacteriol.* **168**, p.1070-1074.

Sugihara, A., Shimada, Y. and Tominaga, Y. (1990). Separation and characterization of two molecular forms of *Geotrichum candidum* lipase. *J. Biochem.* **107**, p.426-430.

Suguira, M., Oikawa, T., Hirano, K. and Inukai, T. (1977). A novel *Geotrichum candidum* lipase with same preference for the 2-position an a triglyceride molecule. *Biochim. Biophys. Acta* **488**, p.353-358.

Suguira, M. and Isobe, M. (1975). Studies on the lipase of *Chromobacterium viscosum* IV. *Chem. Pharm. Bull.* **23**, p.1266-1230.

Suguira, M., Isobe, M., Muroya, N. and Yamaguchi, T. (1974). Purification and properties of a *Chromobacterium* lipase with a high molecular weight. *Agric. Biol. Chem.* **38**, p.947-952.

Suzuki, T., Mushiga, Y., Yamane, T. and Shimuzi, S. (1988). Mass production of lipase by fed-batch culture of *Pseudomonas flourescens*. *Appl. Microbiol. Biotechnol.* **27**, p.417-422.

Switzer, R.C., Merril, C.R. and Shifrin, S. (1979). A highly sensitive silver stain for detecting proteins and peptides in poliacrylamide gels. *Anal. Biochem.* **98**, p.231-237.

Sztajer, H., Borkowski, J. and Sobiech, K. (1991). Purification and properties of lipase from *Penicillium simplicissimum*. *Biotechnol. Appl. Biochem.* **13**, p.65-71.

Taipa, M.A., Aires-Barros, M.R. and Cabral, J.M.S. (1992). Purification of lipases. *J. Biotechnol.* **26**, p.111-142.

Talon, R., Montel, M.C., Grandemer, G., Viau, M. and Cantonnet, M. (1993). Lipolysis of pork fat by *Stapphylococcus waneri*, *S. saprophyticus* and *Micrococcus varians*. *Appl. Microb. Biotechnol.* **38**, p.606-609.

Tanigaki, M., Sekata, M. and Wada, H. (1993). Hydrolysis of soybean oil by lipase with a bioreactor having two different membranes. *J. Ferment. Bioeng.* **75**, p.53-57.

Theil, F. and Björkling, F. (1993). Specificity of *Candida anarctica* Lipase B (SP 435) in the presence of Lipase A in a double enantioselective transesterification. *Biotechnol. Lett.* **15**, p.605-608.

Thirstrup, K., Verger, R. and Carrière, F. (1994). One-step purification and characterisation of human pancreatic lipase expressed in insect cells. *Biochemistry* **33**, p.2748-2756.

Van Oort, M.G., Deever, A.M.T.J., Dijkman, R., Tjeenk, M.L., Verheij, H.M., De Haas, G.H., Wenzig, E. and Gotz, F. (1989). Purification and substrate specificity of *Staphylococcus hyicus* lipase. *Biochemistry* **28**, p.9278-9285.

Van Tilbeurgh, H., Egloff, M.P., Martinez, C., Rugani, N., Verger, R. and Cambillau, C. (1993). Interfacial activation of the lipase-procolipase complex by mixed micelles revealed by X-ray crystallography. *Nature* **362**, p.814-820.

Veeraragavan, K. and Gibbs, B.F. (1989). Detection and partial purification of two lipases from *Candida rugosa*. *Biotechnol. Lett.* **11**, p.345-348.

Veeraragavan, K., Colpitts, T. and Gibbs, B.F. (1990) Purification and characterisation of two distinct lipases from *Geotrichum candidum*. *Biochim. Biophys. Acta.* **1044**, p.26-33.

Verger, R. and De Haas, G.H. (1976). Interfacial enzyme kinetics of lipolysis. *Ann. Rev. Biophys. Bioeng.* **5**, p.77-117.

Virto, M.D., Agud, I., Montero, S., Blanco, A., Solozabal, R., Lascaray, J.M., Llama, M.J., Serra, J.L., Landeta, L.C. and De Rencbales, M. (1994). Hydrolysis of animal fats by immobilised *Candida rugosa* lipase. *Enzyme Microb. Technol.* **16**, p.61-65.

Vora, K.A., Bhandare, S.A., Pradhan, R.S., Amin, A.R. and Modi, V.V. (1988). Characterisation of extracellular lipase produced by *Aspergillus japonicus* in response to *Calotropis gigentea* Latex. *Biotechnol. Appl. Biochem.* **10**, p.465-472.

Wiechelman, K., Braun, R. and Fitzpatrick, J. (1988). Investigation of the bicinchoninic acid protein assay; identification of the groups responsible for color formation. *Anal. Biochem.* **175**, p.231-237.

Winkler, U.K. and Stuckman, M. (1979). Glycogen, hyaluronate, and some other polysaccharides greatly enhance the formation of exolipase by *Serratia marescens*. *J. Bacteriol.* **138**, p.663-670.

Wooley, P. and Petersen, S. (1994). Lipases. Cambridge University Press.

Wu, X.Y., Jääskeläinen, S. and Linko, J-J. (1996). Purification and partial characterization of *Rhizomucor miehei* lipase for ester synthesis. *Appl. Biochem. Biotechnol.* **59**, p.145-158.

Zamost, B.L., Nielsen, H.K. and Starnes, R. L. (1991). Thermostable enzymes for industrial applications. *J. Ind. Microb.* **8**, p.71-82.

# SUMMARY

Lipases (EC 3.1.1.3) or acylglycerol hydrolases, which are widely distributed in nature, are enzymes that catalyse the reversible hydrolysis and synthesis of tri-, di- and monoacylglycerols. These enzymes can be used for chemical modification of lipids, processes such as hydrolysis, ester synthesis and interesterification reactions. Current research has focussed on the determination of the 3D-structure of lipases, industrial application of lipases, immobilisation of lipases, genetic engineering, use of lipases in organic systems and the isolation of new lipases.

Black yeast isolates (23) were obtained from the departmental yeast culture collection of the Department of Microbiology and Biochemistry, UOFS. These isolates were screened for lipase activity on agar plates containing four different inducers. Two isolates were chosen for further studies, namely *Exophiala dermatiditis* UOFS Y-2044 and *Exophiala dermatiditis* UOFS Y-2048. Lipase production during shake culture was determined and optimised using olive oil as inducer.

Isolation of the two extracellular lipases present in the supernatant after centrifugation of the culture medium and the addition of a protease inhibitor, PMSF, included the following steps: cation exchange chromatography on Toyopearl SP-650M, anion exchange chromatography on Toyopearl Super-Q 650S, gel permeation on Toyopearl HW50F and affinity chromatography on MIMETIC Red and Yellow dye ligands for ED2044L and ED2048L, respectively.

The final purification protocol for ED2044L resulted in a 58-fold purification, a specific activity of 1,73 U/mg and a final yield of 42,7%. One band with an approximate molecular mass of 23 600 was visible on SDS-PAGE. The purified ED2044L showed maximal activity under alkaline pH conditions with an optimum pH of pH8,5. The lipase had an optimum temperature of 50°C. The lipase was not thermostable at temperatures higher than 35°C, with an approximate half-live of 3,2 hours at 40°C. EDTA significantly inhibited the activity of ED2044L, indicating that this is a metalloenzyme. Calcium(II), tin(II) and copper(II) inhibited the lipase activity, whereas magnesium(II), iron(III), mercury(II), barium(II) and manganese(II) activated the lipase activity. ED2044L was affected by detergents, with CHAPS, sodium deoxycholate, Cetrimide, Triton-X-100, Tween-80 and SDS inhibiting the lipase activity. PMSF activated the lipase at lower concentrations and inhibited the lipase activity by approx. 30% at higher concentrations. The lipase showed interfacial activation. From the substrate specificity results, it was concluded that ED2044L prefers short chain substrates.

The final purification protocol of ED2048L resulted in a 3-fold purification , a specific activity of 1,6U/mg and a final yield of 13,5%. No bands were visible on SDS-PAGE, because of the low protein concentration. The purified ED2048L showed maximal activity under alkaline pH conditions with an optimum pH of 8,5. The lipase had an optimum temperature of 65°C. The lipase was not thermostable at temperatures higher than 35°C, with an approximate half-live of >24 hours at 40°C, indicating that ED2048L was more thermostable than ED2044L. The lipase showed interfacial activation. From the substrate specificity results, it was concluded that ED2048L also prefers short chain substrates.

Enough evidence to identify the two enzymes as true lipases was provided. The presence of interfacial activation with tripropionin and their activity on triacylglycerol substrates confirmed this.

The aim of this study, namely to isolate and purify two lipases produced by black yeasts and compare them with each other and other lipases, was successfully completed.

# OPSOMMING

Lipases (EC 3.1.1.3) of asielhidrolases, wat wyd verspreid in die natuur voorkom, is ensieme wat die omkeerbare hidrolase en sintese van tri-, di- of monoasielgliserole kataliseer. Lipases kan egter ook gebruik word vir die chemiese modifisering van lipiede asook prosesse soos hidrolise, ester sintese en interesterifikasie. Huidige navorsing behels die bepaling van die 3D-struktuur van lipases, industriële toepassing van lipases, gebruike van lipases in organiese sisteme, asook die volgehoue isolasie van nuwe lipases.

Swart gis isolate (23) is verkry van die departementele gis versameling van die Departement van Mikrobiologie en Biochemie, UOVS. Die isolate is getoets vir lipase produksie deur dit uit te streep op agar plaatjies met vier verskillende induseerders. Twee isolate is gekies vir verdere studies, naamlik *Exophiala dermatiditis* UOFS Y-2044 en *Exophiala dermatiditis* UOFS Y-2048. Lipase produksie is gedurende kultivering bepaal en geoptimiseer deur olyfolie as induseerder te gebruik.

Die isolasie van die twee esktrasellulêre lipases teenwoordig in die bovloeistof na sentrifugering van die kultuur medium en die byvoeging van a protease inhibeerder, PMSF, het die volgende stappe ingesluit: katioon-uitruilingschromatografie m.b.v. Toyopearl SP-650M, anioon-uitruilingschromatografie m.b.v. Toyopearl Super-Q 650S, gelfiltrasie m.b.v. Toyopearl HW50F en affiniteits chromatografie m.b.v. MIMETIC Red en Yellow kleur ligande vir onderskeidelik ED2044L en ED2048L.

Die resultaat van die finale suiwering van ED2044L was 'n 58-voudige suiwering met 'n spesifieke aktiwiteit van 1,73U/mg en 'n finale opbrengs van 42,7%. Een band met 'n relatiewe molekulêre massa van 23 600 was sigbaar op die SDS-PAGE gel. Die suiwer ED2044L het maksimale aktiwiteit onder alkaliese toestande getoon met 'n optimum pH van 8,5. Die lipase het 'n optimum temperatuur van 50°C gehad. Die lipase is nie termostabiel by temperature bokant 35°C en het 'n relatiewe half-leeftyd van 3,2 ure by 40°C getoon. EDTA het 'n noemenswaardige inhibering effek op die lipase gehad, wat impliseer dat die ensiem miskiem 'n metalo-ensiem is. Die lipase aktiwiteit is geïnhibeer deur kalsium(II), tin(II) en koper(II). Magnesium(II), yster(III), kwik(II), barium(II) en mangaan(II) het gelei tot die aktivering van die lipase se aktiwiteit. Die effek van die wasmiddels CHAPS, natrium deoksikolaat, Cetrimide, Triton-X-100, Tween-80 en SDS het gelei tot die inhibering van ED2044L aktiwiteit. PMSF het die lipase aktiwiteit geaktiveer by laer

konsentrasies en daarna by hoër konsentrasies sowat 30% geïnhibeer. Die lipase het skeidingsvlak aktivering getoon. Van die substraat spesifisiteit studies, kan dit afgelei word dat die lipase kort ketting substrate verkies.

Die resultaat van die finale suiwering van ED2048L was 'n 3-voudige suiwering met 'n spesifieke aktiwiteit van 1,6U/mg en 'n finale opbrengs van 13,5%. Geen bande was sigbaar op die SDS-PAGE gel nie, heel waarskynlik toe te skryf aan die baie lae proteïen konsentrasie van die lipase. Die suiwer ED2048L het maksimale aktiwiteit onder alkaliese toestande getoon met 'n optimum pH van 8,5. Die lipase het 'n optimum temperatuur van 65°C gehad. Die lipase is nie termostabiel by temperature bokant 35°C en het 'n relatiewe half-leeftyd van >24 ure by 40°C getoon, wat impliseer dat ED2048L meer termostabiel is as ED2044L. Die lipase het skeidingsvlak aktivering getoon. Van die substraat spesifisiteit studies, kan dit afgelei word dat die lipase kort ketting substrate verkies.

Voldoende bewyse om die twee ensieme as egte lipases te identifiseer is gevind. Die voorkoms van skeidingsvlak aktivering met tripropionien en die aktiwiteit van die ensieme met twee triasielgliserol substrate was 'n bevestiging hiervan.

Die doel van die projek, naamlik die isolasie en suiwering van twee lipases wat deur swart giste geproduseer is, en die vergelyking van die twee lipases met mekaar en ander lipases, is suksesvol afgehandel.

Buiteblad:

<u>Exophiala dermatiditis</u> lipase:
Isolation and characterisation

J. Hamilton

Kant:

J. Hamilton 1998

U.O.V.S. - U.O.F.S.
BLOEMFONTEIN
BIBLIOTEEK - LIBRARY